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The Journal of Biochemistry, Vol. 44, No. 3, 1957

TURBIDOMETRIC TITRATION OF SERUM-PROTEIN

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(Received for publication, September 5, 1956)

Methods for the fractionation and analysis of serum protein have been reported by many investigators. These methods can be classified into following four groups according to the physicochemical mechanism involved, namely, method using difference in solubility, electrophoretic method and electrophoresis convection method, ultracentrifugal method, and chromatographic method. Recently, methods of subfractionation of serum protein such as Cohn's alcohol fractionation (*1*, *2*) method, Derrien's method (*3*, *4*), Sober's chromatographic method (*5*), Kirkwood's electrophoresis convection method (*6*, *7*), and improved electrophoretic method (*8*, *9*), have been reported.

It is well known that serum proteins are precipitated by the addition of neutral salts, such as ammonium sulfate (*10*), sodium sulfate. With the use of these phenomena, the present authors have tried to devise a convenient method by which continuous observation can be carried out. For this purpose, the authors have investigated the differential salting-out of protein by using ammonium sulfate as a precipitant and devised a convenient turbidometric analysis of protein mixture (*11*, *12*). Let him be called, " turbidometric titration." Even in the case of volume of serum as small as 0.5 ml., the turbidometric titration of human-serum was easily established.

The authors first obtained twelve components (*13*) for the human-serum-protein, say, a′, a, b, c, . . . h, i, j, k. The twelve components were classified into four groups, say globulin I (a′, a, b, c), globulin II (d, e), globulin III (f, g) and albumin (h, i, j, k). The turbidometric diagram of those four groups exactly resembles the familiar electrophoretic ones. Furthermore, the authors (*14*, *15*) obtained twenty components for rabbit-serum-protein, five components for human-serum-albumin, and so on for many others.

EXPERIMENTAL

The apparatus essentially consists of a glass-cell with the capacity of 40 ml., fitted with stirrer rotating at 100 r.p.m., and a burett above the surface, through which the saturated ammonium sulfate solution drops into the protein solution. The beam of light passing through the cell is received by a photocell of an electrophotometer.

It is desirable that the cell of the electrophotometer be equipped with a thermostate. But this can be omitted when ammonium sulfate solution as a precipitant, as we did, because the salt does not precipitate under room temperature.

A. General Method

First, serum is diluted with 1 per cent NaCl solution from ten-fold to twenty-fold. An accurately measured volume of the diluted solution is put into the glass-cell. Saturated ammonium sulfate solution (precipitant) is added from microburett to 16.00 ml. protein solution under constant stirring. After adequate interval, the stirrer is stopped and the turbidity of solution is measured. And then, the above mentioned procedures are repeated. In each stage of titration, an adequate interval is required before the true turbidity of solution is obtained.

After completion of titration, the concentration of salted-out protein in every step is calculated by the help of the Lambert-Beer's equation (*11*, *12*, *13*, *16*), namely equation (1):

$$1\,\mathrm{n}\ I_{\circ}/I=kS \qquad (1)$$

where $I_{\circ}$ and I are intensities of transmited beam through solvent and protein suspension respectively, k is a proportional constant and S stands for the concentration of the salted-out protein. Since the already precipitated protein is continuously diluted with the addition of precipitant, it is necessary to correct S to some fixed value. It is, however, easy to obtain its corrected value T, if one uses the following equation:

$$kS\times\frac{V_i}{V_{\circ}}=T \qquad (2)$$

where $V_{\circ}$ is the original volume of the protein solution and V_i is the volume in each step of the titration. And then, the difference between T before and after each titration, $\Delta T/\Delta C$, is plotted against the concentration of ammonium sulfate, as shown in Figs. 1, 2, . . . , and these figures are called " turbidometric diagram " by authors.

The Protein-content—The initial protein content should be kept low (0.3, 0.6 per cent in the case of serum protein) for the following reasons.

(1) The equation (1) is applicable only to a limited concentration range of protein.

(2) In every step of titration, a relatively long interval is necessary to reach the true turbidity of solution but this can be shortened by decreasing protein content.

The NaCl-content—(1) At the same protein level, though salted-out protein increases according to the concentration of ammonium sulfate, the turbidity of the solution is not proportional to the salted-out protein; namely, the turbidity increases until a certain value and then make a decreasing curve. Though this phenomenon was a disturbance in turbidometric titration, it was excluded to some extent by decreasing the concentration of protein and almost completely by addition of a small amount of Cl^-, namely 1 per cent NaCl. Therefore, 1 per cent NaCl solution is used as solvent for protein. This improvement is very important to the turbidometric titration.

The turbidometric titration of synthetic polymers has recently been investigated by D. R. Morey *et al.* (*16, 17*), I. Harris *et al.* (*18*), A. S. Dunn *et al.* (*19*) and H. W. Melville (*20*). They have excluded this difficulty by decreasing the concentration of high polymer.

B. Specific Method to obtain Finer Turbidometric Diagram

When the turbidometric titration is limited to a particular protein fraction, such as γ-globulin, the serum is dissolved in a 25 per cent ammonium sulfate solution and is titrated with 75 per cent ammonium sulfate solution. Then, more refined results are obtained. Example of such curve is shown in Fig. 7.

C. Specific Method to establish the Turbidometric Titration in the Case of Lower Protein-content

In the case of lower protein-concentration, the protein solution is salted-out by ammonium sulfate and is centrifuged to concentrate the salted-out protein. The precipitates are pippeted out, diluted to optimal protein concentration with 1 per cent NaCl solution and then titrated with ammonium sulfate solution of an arbitary concentration. Hence, specific concentrating methods, such as freezing-drying or ultrafiltration, are not necessary.

RESULTS

The results of human-serum-protein are shown in Figs. 1 and 2. where the way of obtaining serum is different. The human-serum-protein was fractionated into twelve components, say, a′, a, b, c, . . . i, j, k, as shown in Figs. 1 and 2. These diagrams are similar to those of Derrien and Steyns-Parvé. It was found that the diagrams of the human-serum-albumin vary according to the way of obtaining serum as shown in Figs. 1 and 2.

The reproducibility of the turbidometric diagram turned out quite satisfactory as shown in Figs. 3 A and 3 B, where the material was the human-serum-protein. It is clear from these diagrams that peaks are not caused by experimental errors.

When the difference quotient is evaluated, ΔC is taken on a larger scale than in previous one in order to classify these refined components. Then, human-serum-protein was classified into four-groups, say, serum-globulin I, II, III, serum-albumin, as shown in Fig. 2-B. Thus, the twelve components are classified into four groups, say, globulin I (a′, a, b, c), globulin II (d, e), globulin III (f. g), albumin (h, i, j, k).

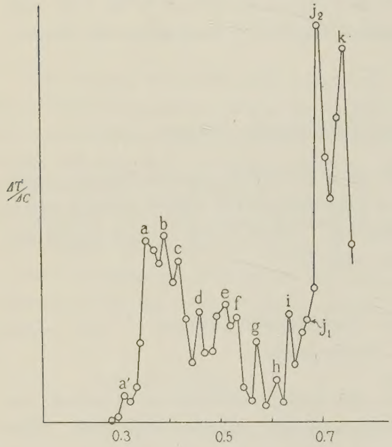

FIG. 1. The turbidometric diagram of human-serum-protein. The abscissa and the ordinate show the volume fraction, C, of saturated ammonium sulfate solution and $\Delta T/\Delta C$, respectively, where T is the relative amount of salted-out protein. Experimental conditions were as follows: protein concentration 0.5 per cent, pH 6.3 at 30 per cent saturation and 5.9 at 70 per cent saturation.

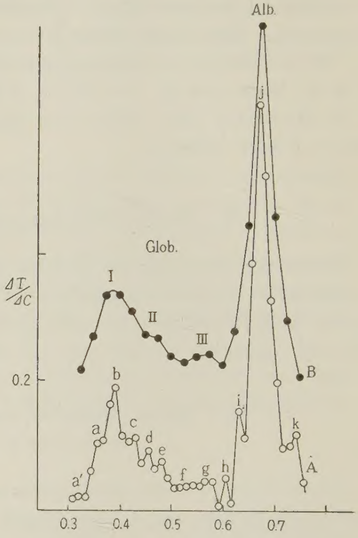

FIG. 2. The turbidometric diagram of human-serum-protein. The diagram (A) and (B) show the twelve components and four groups of human-serum-protein, respectively. Diagram (B) is made by smoothing out diagram (A), *i.e.*, taking larger ΔC, in (B) than in (A) by a certain amount so as to give four groups instead of twelve. Experimental conditions were as follows: protein concentration 0.5 per cent, pH 6.3 at 30 per cent saturation and 5.9 at 70 per cent saturation.

Making some technical improvement on the first experiment, rabbit-serum-protein was fractionated into about twenty components as shown in Fig. 4 ; human-serum-albumin into five components, say, h, i, j_1, j_2, k, as shown in Fig. 5 ; human-globulin I into seven components, say, a′, a_1, a_2, b_1, b_2, c_1, c_2, as shown in Fig. 7.

When the turbidometric titration is limited to the particular protein fraction such as globulin—I, the more refined diagram was obtained easily by the above mentioned method (B). Namely, the rabbit-serum-protein is dissolved in 25 per cent saturated ammonium sulfate solution and then titrated with 75 per cent saturated ammonium sulfate solution. Both the ammonium sulfate solutions are diluted with use of 1 per cent NaCl solution. These results are shown in Fig. 7.

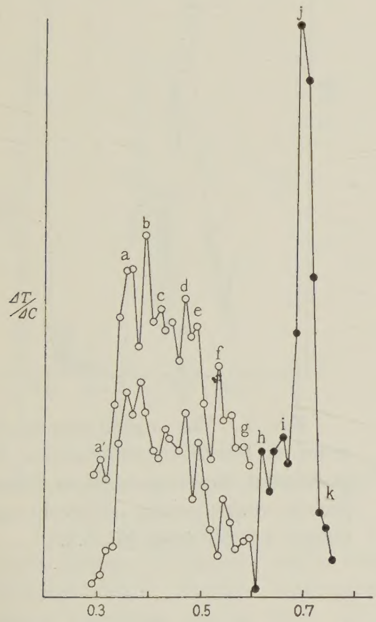

FIG. 3 A. The turbidometric diagram of human-serum-protein. Experimental conditions were as follows: protein concentration 0.6 per cent, pH 6.3 at 30 per cent saturation and 5.9 at 70 per cent saturation.

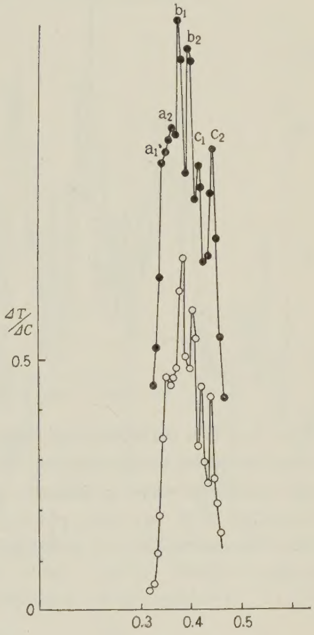

FIG. 3 B. The turbidometric diagram of human-serum-γ-globulin. Experimental conditions were as follows: protein concentration 0.20 per cent, pH 5.30.

The turbidometric titration of the lower protein-content is easily established by the help of method (C). The turbidometric diagram obtained by this method is entirely similar to Fig. 7 and is shown in Fig. 8.

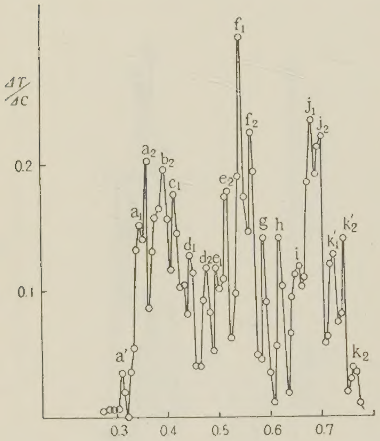

FIG. 4. The turbidometric diagram of pathological rabbit-serum-protein. Experimental conditions were as follows: protein concentration 0.35 per cent, pH 6.3 at 30 per cent saturation and 5.9 at 70 per cent saturation.

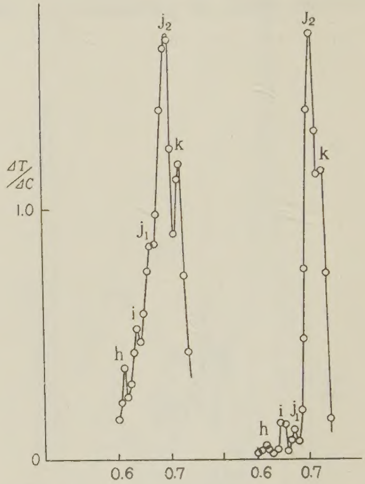

FIG. 5. The turbidometric diagram of human-serum-albumin. Experimental conditions were as follows: protein concentration (albumin+globulins) 0.6 per cent, pH 5.9.

DISCUSSIONS

The authors' experimental method and results were explained briefly in the last two sections. In this section, the authors' method will first be made comparison with other methods, and then merits and demerits of the authors' method will be discussed.

The refined fractions of serum-protein were obtained by the improved electrophoretic method, namely Kirkwood's electrophoresis convection (*6*, *7*) and Seifer's method (*8*, *9*). A. Seifer and E. J. Corey (*8*, *9*) observed the subfraction of human-serum-albumin, electrophoretically. The essential point of their method is to electrophorate the so called electrophoretically purified serum albumin at a lower ionic strength. Then, serum-albumin was fractionated into three or four components. However, this method is more complicated than the authors' one in which purification is not necessary. Though electrophoretic method has been commonly used and has brought great advances in protein chemistry, it will be difficult to fractionate finely the serum-protein by the ordinary electrophoresis except for Seifer's method, since the concentration gradient of protein formed by applied electric field would be decreased by the diffusion of the protein.

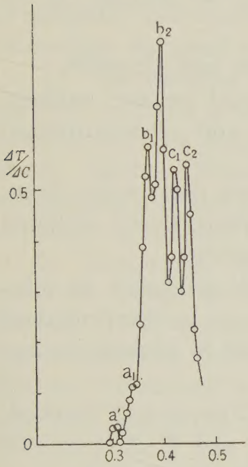

FIG. 6. The turbidometric diagram of human-serum-globulin I. Experimental conditions were as follows: protein concentration (albumin+globulins) 0.6 per cent, pH 6.3.

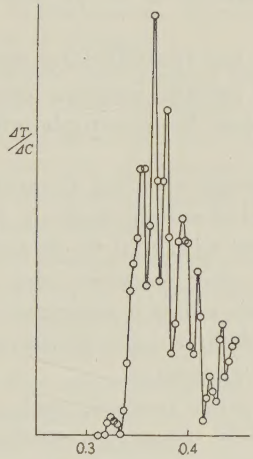

FIG. 7. The turbidometric diagram of rabbit-serum-globulin I (Method B).

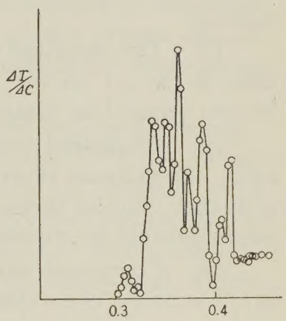

FIG. 8. The turbidometric diagram of rabbit-serum-globulin I (Method C).

In the case of using the difference in solubility or chromatographic method, the above mentioned factor does not decrease the fineness of results. Therefore, the methods using the difference in solubility is more conducive to fine results. Derrien (*3*) and Steyn-Parvé (*4*) obtained refined fractions of serum-protein with the use of salting-out and by Kjeldahl's nitrogen analysis. The authors' turbidometric diagrams are very similar to Derrien's results. Fig. 2-B conforms to the results obtained by Amirkhanova *et al.* (*21*) with the use of the salting-out and the centrifugal separation of salted out protein. The subfractionation of serum-protein could probably be obtained by S. N. Amirkhanova's method in the case of small quantity of serum. Cohn *et al.* (*1, 2*) investigated the fine fractionation of serum-protein controlling the four variables, namely pH, ionic strength, dielectric constant and temperature, and obtained very fine results. A number of Cohn's fractions are similar to authors' but it should be noted that there is no one-to-one correspondence between them. H. A. Sober *et al.* (*5*) obtained fine fraction of serum protein by making use of chromatographic method.

The authors' method has the following merits and demerits.

(1) The number of protein fractions obtained by the authors' method is larger than those by electrophoretic and ultracentrifugal method.

(2) The standard deviation of the Gaussian distribution function, N (m, σ), of the turbidometric diagram is more easily obtained by our method as compared with that of electrophoresis.

(3) Compared with other methods using the difference in solubility, the quantity of protein which is necessary for the authors' method is smaller than for them. And, it might be decreased by making further improvements on authors' apparatus.

(4) Compared with other method, such as Derrien's method, Kirkwood's electrophoresis convection method and H. A. Sober's chromatographic method, our procedure is not time consuming.

(5) Even in the case of lower concentration of protein, the turbidometric titration is easily established as compared with the electrophoretic method, since in the latter, freezing-drying or ultrafiltration is necessary to concentrate the protein.

(6) The apparatus is not costly and can be made easily.

As stated above, the authors' method has many merits. However, the following problems must be deservedly improved, that is, although the authors assumed that the constant k in equation (1) is equal in all of the protein-fraction, the k will not probably be constant. And also, the following fundamental experiments must be established.

(1) An effect of pH on the turbidometric diagram. (In this report, pH changed as follows ; 6.3 at 30 per cent saturation, and 6.0 at 75 per cent saturation.)

(2) An effect of temperature on the turbidometric diagram.

(3) An effect of various anions of buffer on the turbidometric diagram.

(4) Whether or no every peak in the turbidometric diagram represents the real protein-fraction.

(5) The relation between electrophoretic and turbidometric diagrams.

SUMMARY

A very sensitive method for fractionating the serum-protein was investigated and called turbidometric titration by the authors. The conclusions obtained were as follows:

1. The essential point of this method lies in making use of turbidometric analysis combined with salting-out. Ammonium sulfate solution is used as a precipitant and 1 per cent NaCl solution as solvent for serum. Authors found that the concentration of the protein should be kept low and the protein-solution should contain NaCl, these being necessary to achieve reliable results.

2. The human-serum-protein was fractionated into twelve components, say, serum albumin (h, i, j, k), serum-globulin III (f, g), serum-globulin II (d, e), and serum-globulin I (a′, a, b, c). Making some improvements on the first experiments, it was found that human-serum-albumin is fractured into five components (h, i, j_1, j_2, k), human-serum-globulin I into seven components (a′, a_1, a_2, b_1, b_2, c_1, c_2) and rabbit-serum-globulin I into eight components.

3. Even in the case of low protein content, the turbidometric titration was performed easily without specific concentrating methods such as freezing-drying or ultrafiltration.

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SPECTROSCOPIC STUDIES ON CHLOROPHYLL FORMATION IN INTACT LEAVES

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(Received for publication, October 23, 1956)

Recently, Smith and co-workers (*1*, *2*, *3*) have extracted protochlorophyll *a* in what appears to be its natural state, and Smith and Kupke (*4*) have since identified the extracted protochlorophyll activity with a homogeneous appearing fraction in the ultracentrifuge. They have tentatively referred to these active particles as protochlorophyll *a* holochrome (*5*). The extracted protochlorophyll had absorption maxima between 635 and 650 mμ, approaching the longer wavelength when the preparations were most active. By light, the protochlorophyll in these extracts was transformed into chlorophyll *a*, which had an initial absorption maximum near 680 mμ. They also observed a progressive shift of the newly formed chlorophyll *a* band from 680 mμ to 672 mμ, when the sample was kept in the dark at room temperature for 3 hours (*3*). Active extracts of protochlorophyll have recently been reported also by Krasnovskii and Kosobutskaya (*6*), although their medium for extraction was different from that used by Smith *et al.* According to Krasnovskii *et al.*, a colloidal solution of protochlorophyll *a* in its natural state has an absorption band at 635 mμ, and it is transformed into chlorophyll *a*, which has an absorption peak at 670 mμ. After the etiolated leaf has been illuminated for some hours, the absorption band of the colloidal extract shifts to longer wavelengths, 677–678 mμ. Krasnovskii *et al.* attributed the shift to the change from a monomeric form of chlorophyll *a* to the colloidally aggregaged form. While the protochlorophyll *a* holochrome system isolated by both groups of scientists can be transformed rather efficiently, the direction of the wavelength shift of the chlorophyll *a* band with time is different. Furthermore, the bands of both protochlorophyll *a* and the initially formed chlorophyll *a* are found at different positions by these groups, as they should not be, if both of the samples are in the same natural state. If we wish to consider the naturally occurring process, it is desirable to examine intact materials rather than extracts thereof.

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The difficulties, that have, thus far, prevented observation of the absorption spectra of protochlorophyll in intact leaves, have been the low pigment content and the light scattering of the leaf. The light scattering, particularly, obscures the true absorption spectrum of the leaf. Shibata, Benson and Calvin (*8*) have now developed a new method for obtaining clear absorption spectra of translucent, light scattering materials by use of opal glass. This method was used in this study, to observe the absorption spectra of intact etiolated leaves and the changes in these spectra when the protochlorophyll in the leaves was changed to chlorophyll by illumination. As will be shown later, the observations of the absorption spectra of etiolated leaves and their changes by illumination not only answered the questions about the positions of the maxima of protochlorophyll and chlorophyll *a* derived therefrom in the intact leaf, but also revealed that native chlorophyll initially formed from protochlorophyll undergoes several changes before reaching its final state.

We propose to designate the various protochlorophyll and chlorophyll found *in vivo* by the appropriate position of the major absorption peak. This system was introduced by Duysens (*7*) for the various bacteriochlorophyll found *in vivo*. In this system, the letter designates the compound found on extraction; P=protochlorophyll, C=chlorophyll *a* and the number refers to the red peak position of the *in vivo* complex. But as will be explained later, there are three kinds of chlorophyll-*a*-like substances involved in the course of the formation of chlorophyll *a*. The identification of those compounds as chlorophyll *a* was carried out only by the red peak *in vitro*. Therefore, in the case of the usage of C as " chlorophyll *a* ", it means the substance very similar to chlorophyll *a*, but it could be slightly different in the state or chemically from chlorophyll *a in vitro*.

METHOD

The observations were carried out with a Beckman DK-2 recording spectrophotometer. Etiolated leaves were sandwiched in the dark, between a sheet of opal and a sheet of transparent glass, and were placed in the compartment of the spectrophotometer. The same two kinds of glass without the sample were used for the reference light beam (*8*). From one to three superimposed leaves were used for the observations of the absorption spectra, depending on the spectral range and the purpose of the experiment. The intensity of the measuring beam was too weak to transform protochlorophyll *a*.

As will be shown later, the etiolated leaves have high absorption bands in the Soret region. For the observation of these bands, the reference beam was partly masked with black tape in order to keep the recorder scale in the range between 0 and 1 absorbance units. In other words, the zero point was shifted down by masking the reference beam instead of using the electrical scale shifting device in the spectrophotometer. This method reduces the noise and provides a broader proportional range of absorbance than does the electrical device. In this way, we can observe absolute absorbance values of greater 2.

In this study, the changes in the absorption spectra are more important than are the absolute values of the absorbance. To observe small changes, the signal in absorbance units from the spectrophotometer was electronically amplified to as much as twice the original signal. In order to observe large changes or a broader range of the spectrum, the signal was reduced to one half.

By varying the number of layers of leaves, the amplification of the signals, and zero level shift, it was possible to make the changes of the leaf spectra cover the full range of the recorder scale, independently of the absolute height of the spectrum. The spectra shown in this report, except for those in Figs. 1 and 2 are not expressed in absolute units of absorbance. The deviation from the absolute value caused by amplification of the signals was roughly corrected, but the shifting of the zero point was not corrected, so that the value of absorbance shown beyond Fig. 2 is the absolute value of absorbance minus a constant for each figure.

In the Soret region, where the absorption curve rises steeply, the change of the spectra caused by illumination of the leaf was difficult to estimate by direct observation of the absorption spectra. In such cases, the difference in the absorption spectrum of the illuminated and unilluminated leaves (difference spectra) was recorded instead of the absorption spectrum itself. This permits the observation of the spectrum in the entire visible region in a short period without masking the reference beam, although the results are more difficult to interpret than the usual absorption spectra. Two sheets of leaves were sandwiched between two transparent glasses for each beam for the observation of difference spectra. In this case, opal glass was not necessary. This is because one of the two sheets of leaves acts as an effective diffuser of light much as would a sheet of opal glass. We can use a narrower slit width in this way than with an additional sheet of opal glass. This system is, of course, used only with highly diffusing materials such as two sheets of bean leaves in both the sample and reference beams.

In this study, etiolated leaves of *Phaseolus vulgaris* var. Ferry-Morse's Pole bean No. 191 were mostly used. A few experiments with etiolated *Zea Mays* (corn) leaves are also reported. The leaves came out of the cotyledons about a week after planting. The leaves were picked in the darkroom with the aid of a green light, which is weak enough not to transform protochlorophyll *a* into chlorophyll *a*. For a few days after the leaves come out of the cotyledons, they are soft and slippery on the surface. During the next few days, they become larger and crisper. Further culturing in the dark makes the leaves fragile so that they can easily be torn along the midrib. Leaves in these stages of development were designated as young, normal, and old leaves, respectively.

The sample was illuminated inside the compartment of the spectrophotometer by a 150 W incandescent lamp as a white light source. The position of the lamp was adjusted to obtain either 1000 foot-candles or 300 foot-candles on the surface of the sample. The illumination by monochromatic light of 650 mμ was carried out in a darkroom by the light from a 1000 W tungsten lamp, which then passed through a large grating monochromator which was built by French (*9*). Corning filter No. 2403 was set in front of the sample to eliminate the stray light from the monochromator. The half band-width of the monochromatic beams was 10 mμ.

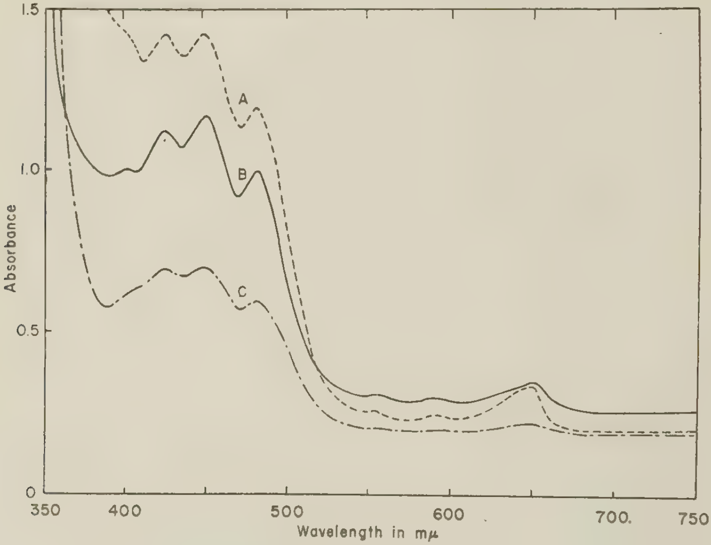


FIG. 1. Absorption spectra of etiolated leaves in the visible region. Each curve represents the spectrum of a single leaf. Curves A and B, young etiolated bean leaf: Curve C, etiolated corn leaf.

RESULTS AND DISCUSSION

1. The Absorption Spectra of Etiolated Leaves

In Fig. 1, absorption spectra of a single layer of etiolated leaves for the whole visible region are shown. Curves A and B are the spectra of young etiolated bean leaves from different cultures, and curve C is the spectrum of an etiolated corn leaf. Absorbance is expressed in absolute units. We can see a great difference in the height of the spectra even in the young bean leaves (curves A and B), but the positions of the absorption maxima are the same. The amount of the pigments per unit area in the corn leaf is about half that in the bean leaves. In the Soret region, there are three high absorption bands at 481, 449 and 425 mμ with a lower band at 400 mμ. From the shape of the three high peaks, they appear to be due to absorption by carotenoids. However, from the experiments shown later, the Soret band of protochlorophyll *a*, P 650, was found to be at 445 mμ. Therefore, the second band at 449 mμ is probably due to the presence of both protochlorophyll *a* and carotenoids.

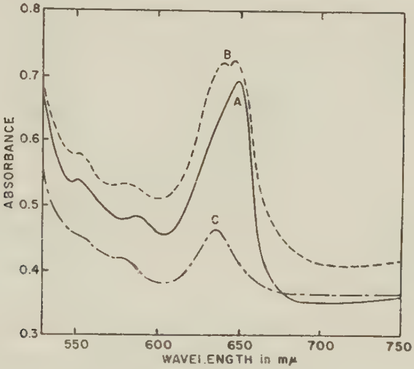


FIG. 2. Absorption spectra of etiolated leaves in the red region. Each curve represents the spectrum of two superimposed leaves. Curve A, young etiolated bean leaves: Cuve B, old etiolated bean leaves: Curve C, bleached old etiolated bean leaves.

To see the spectrum in the red region more clearly, two layers of young etiolated bean leaves were observed in the region between 530 and 750 mμ (curve A in Fig. 2). The spectrum shows a sharp peak at 650 mμ with low bands at 590 and 555 mμ. The position of 650 mμ agrees with the value that Smith *et al.* (*3*) have observed, but does not agree with the value of 635 mμ obtained by Krasnovskii *et al.* (*6*). It does agree with the position, 650 mμ, which Koski, French and Smith (*10*) have observed in the action spectrum for the transformation of protochlorophyll *a* to chlorophyll *a*, although Schmidt (*11*) gave the value of 640 mμ and Frank (*12*) gave the value of 654 mμ in their action spectra. According to Koski *et al.* (*10*), the action spectrum has low peaks at 593 and 550 mμ beside this red band and the high Soret peak at 445 mμ. These low bands also agree fairly well with our data ; 590 and 555 mμ.

The data with young etiolated leaves seem to be consistent with the data, that have been published so far. The spectrum of old etiolated bean leaves is more complicated ; namely, the spectrum (curve B in Fig. 2) shows a double peak slightly below 650 mμ in place of a single peak at 650 mμ as observed in young etiolated leaves, and the position of one of the low bands is 582 mμ instead of 590 mμ. From the comparison of curves A and B, it is likely that there is another band which is predominant in old leaves and has an absorption band around 635 mμ. The position of this peak was more accurately measured in the following way. Old etiolated leaves were heated in water at 60° for a few minutes in the dark, and then illuminated with strong light (1000 foot-candles) for 10 minutes. Curve C in Fig. 2 shows the resultant spectrum, which has bands at 636, 580 and 555 mμ. This treatment caused the band at 650 mμ and the chlorophyll *a* band formed from protochlorophyll *a* to disappear. Only the band in question at 636 mμ remained as a high peak. From these results, it is clear that in old etiolated leaves there are two components with main absorption bands at 650 and 636 mμ, respectively. They are designated P 650 and P 636. The shape of the peak at 650 mμ of curve A indicates that even the young leaves may contain a small amount of the P 636 component.

A problem arises as to which bands are due to protochlorophyll *a*. From the action spectra referred to above, it is almost certain that the band at 650 mμ is due to protochlorophyll *a*, and the experiment, which is shown in Fig. 3, more clearly supports this interpretation. Curve A in Fig. 3 is the spectrum of two old etiolated bean leaves. This sample was subjected to successive illumination with white light of 300 foot-candles. We can observe the reduction of the band only at the longer wavelength side of the double peak near 650 mμ with the formation of a peak near 680 mμ. Therefore, only the component, that has an absorption band at 650 mμ, is transformed. This means that the band at 636 mμ is not due to active protochlorophyll *a*. Since the culturing in the dark especially for a long time is not the natural process, this component could be due to protochlorophyll *a* inactivated by the artificial conditions. In this connection, Kupke (*16*) has observed that protochlorophyll extracts that have become inactive show that a shift in the absorption maximum to shorter wavelengths of 630–635 mμ has occurred (personal communication). The position of the protochlorophyll maximum in extracts relative to 650 mμ is indicative of the proportion of active protochlorophyll therein.

In the experiments on the phototransformation of protocholrophyll to chlorophyll in the living dark-grown leaf, Smith, Benitz, Koski and Virgin (*13*, *14*, *15*) have always found that the organic-solvent extracts of the illuminated leaves contained residual protochlorophyll. This residuum persisted regardless of the period of illumination. The experiments reported in this paper provide an adequate explanation for this observation. They demonstrate that etiolated leaves contain two protochlorophyll holochromes, distinguishable by spectrophotometric measurements. One of them is readily transformed by light to chlorophyll; the other is not. It is undoubtedly the latter that accounts for the residual protochlorophyll in the leaf extracts examined by Smith and his co-workers.

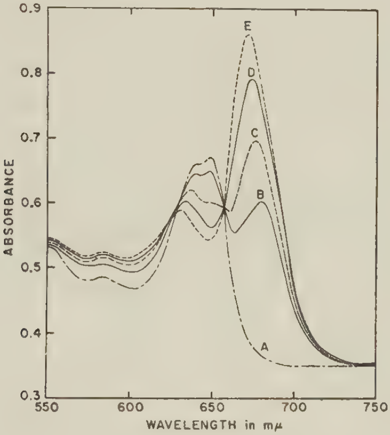


Fig. 3. Effect of successive illumination periods on the spectrum of old etiolated bean leaves. Curve A, two superimposed old etiolated bean leaves before illumination: Curve B, C, D and E, after 0.5, 1.0, 2.5 and 12.0 minutes total illumination, respectively.

2. *The Changes of Etiolated Leaf Spectra in the Red Region due to Illumination by White Light*

As was shown above, there are some differences between the spectra of young and old etiolated leaves. The differences are more easily seen by examining the changes of absorption spectra caused by illumination. In young etiolated leaves, the reaction is much faster than in old leaves. In old etiolated leaves, all reactions proceed so slowly that some of them can hardly be observed. Therefore, young and normal leaves were chosen in order to see the various steps in the formation of chlorophyll. The experiments were carried out by following the changes of the absorption spectra in the dark after a short illumination by strong light (1000 foot-candles) from an incandescent lamp. In this section, the changes of the spectra in the red region are described.

The results obtained with two layers of normal etiolated bean leaves are shown in Figs. 4 and 5. Curve A in Fig. 4 is the spectrum of the original leaves. After illumination for 1 minute, the protochlorophyll *a* band at 650 mμ disappeared and a band at 684 mμ appeared (curve B). To simplify the further explanation, this rapid photochemical change is designated as the first step, P 650 $\xrightarrow{\text{light}}$ C 684.

This illuminated sample was then kept in the dark in the spectrophotometer and the successive dark reactions were followed by measuring its absorption spectrum at intervals. Curves B to G in Fig. 4 show that the band at 684 mμ, which was formed immediately on illumination, slowly decreased in the dark and a band at 673 mμ appeared during a 14 minutes period. In this period, a marked isobestic point was observed at 680±1 mμ. The sharp isobestic point indicates that this process is the transformation from a single compound to another single compound. This transformation in the dark from the 684 to the 673 mμ peak is called the second step, C 684 $\xrightarrow{\text{dark}}$ C 673.

The further changes of the spectrum of this sample in the dark are shown in Fig. 5, in which curve G of Fig. 4 is reproduced. Between 14 and 36 minutes (curve G to I), there was an increase of absorption over the whole range, but the peak at 673 mμ stayed at the same position. The changes in this period are probably due to the overlapping of the second step and the follow-up step shown in the next.

After 36 minutes (curves I to K in Fig. 5), different kinds of changes are more clearly evident. We can see the great increase near 650 mμ caused by the formation of protochlorophyll *a*, Precurser $\xrightarrow{\text{dark}}$

P 650. At the same time, the peak at 673 mμ has gradually shifted to the longer wavelength of 677 mμ after 106 minutes, which agrees with the positions of the peak of chlorophyll *a* in normal green leaves or algae at 676–678 mμ (8). No isobestic point was observed in this shifting process. This shift to 677 mμ is designated as the third step, C 673 $\xrightarrow{\text{dark}}$ C 677. Also during this time, protochlorophyll *a* was formed.

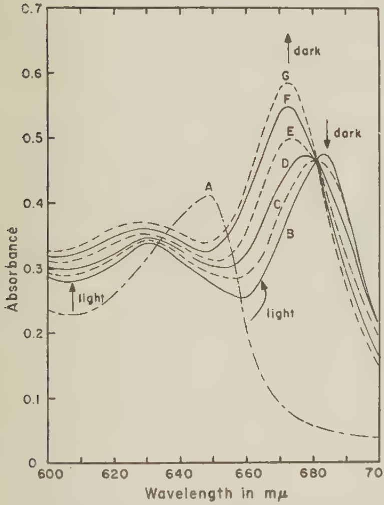


FIG. 4. Change of the spectrum of normal etiolated leaves by illumination with white light (1 minute, 1000 foot-candles). Curve A, two superimposed normal etiolated leaves before illumination: Curves B, C, D, E, F and G, after 0, 3.3, 6.0, 8.8, 11.5 and 14.0 minutes in the dark after illumination, respectively.

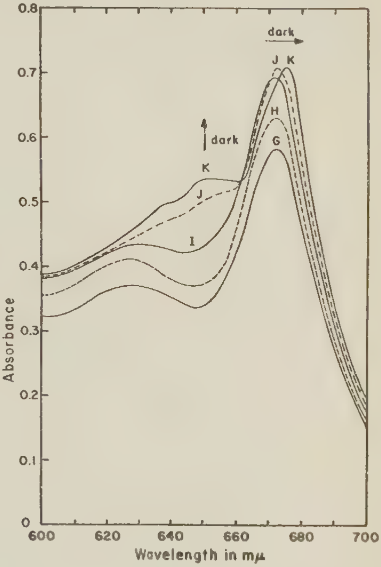


FIG. 5. Further changes of the spectrum of the leaves of Fig. 4 after longer times in the dark. Curves G, H, I, J and K, after 14, 19, 36, 61 and 106 minutes in the dark after illumination.

To make sure of the absorption maximum of the photochemically produced compound by the first step, the illumination period by the 1000 foot-candle light was changed. Reducing the period of illumination to 10 seconds did not change the position of the 684 mμ peak, but increasing the period above 1 minute made the peak wavelegnth shorter, owing to the overlapping of the first and second steps.

A pre-illumination experiment similar to that of Figs. 4 and 5 was carried out, using three etiolated corn leaves. The same steps shown above were also observed in the corn leaves.

In the next experiment, three young etiolated bean leaves were used instead of normal etiolated ones. The experimental conditions were the same as that of the experiment shown in Figs. 4 and 5, although the measured spectral range was extended to 750 mμ. The results are shown in Figs. 6 and 7. The following differences between the reactions in young and normal etiolated leaves were obserevd.

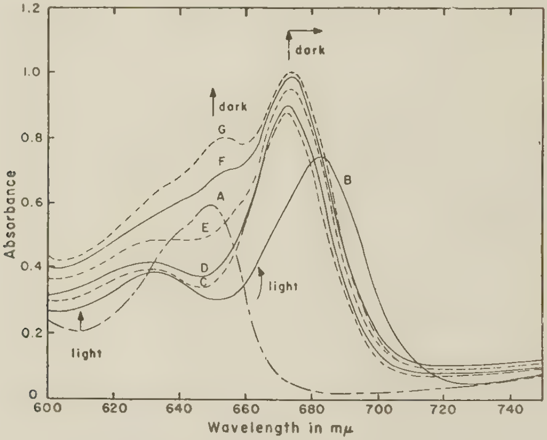


FIG. 6. Change of the spectrum of young etiolated leaves by illumination with white light (1 minute, 1000 foot-candles). Curve A, three superimposed young etiolated leaves before illumination: Curves B, C, D, E, F and G, after 0, 10, 20, 40, 60 and 100 minutes in the dark after illumination.

In young leaves, the second step, C 684 $\xrightarrow{\text{dark}}$ C 673, was nearly complete within 10 minutes (curves B and C in Fig. 6). This rate therefore is considerably faster in young leaves than in normal leaves. The position of the peak after the second step was 671–672 mμ instead of 673 mμ as in normal leaves. After 20 minutes in the dark, the re-formation of protochlorophyll *a*, Precurser $\xrightarrow{\text{dark}}$ P 650, had already started in young leaves (curve D in Fig. 6), and almost ended after 100 minutes (curve G in Fig. 6). The absorption band of P 650 appeared as a definite peak. The shift of the band at 671–672 mμ toward longer wavelengths took place as before, although the position of the peak after 100 minutes was 674 mμ instead of 677 mμ as in normal leaves after the corresponding dark period. It was found from several experiments with different young leaves that the final position of the band varied in the range of 674–676 mμ and was lower than the 677 mμ position in normal leaves.

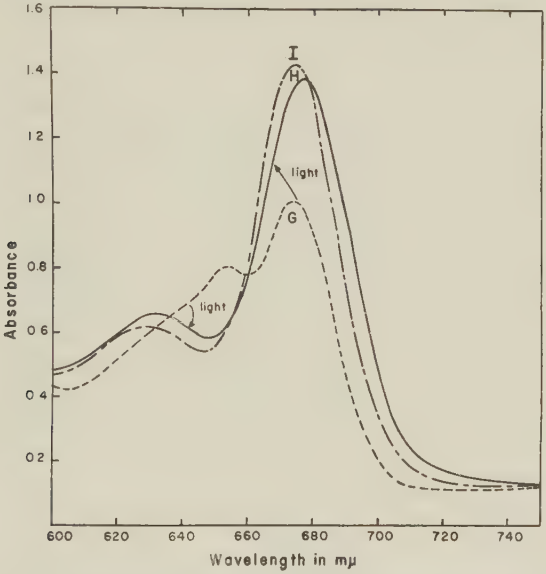


FIG. 7. Further changes in the spectrum of the leaves of Fig. 6 after second illumination. Curve G, 1 minute light +100 minutes dark: Curve H, 1 minute light +100 minutes dark +1 minute light: Curve I, 1 minute light +100 minutes dark +1 minute light +10 minutes dark.

The sample kept 100 minutes in the dark (curve G in Figs. 6 and 7) was illuminated again under the same conditions as before. The band around 650 mμ disappeared and the band at 674 mμ was raised and shifted to 677 mμ (curve H in Fig. 7), which must be due primarily to the overlapping of the newly formed band at 684 mμ and the band already present at 674 mμ. In the next 10 minutes in the dark, the change due to the second step can be observed again (curve I in Fig.

7). The further changes in the spectrum appeared to be the same as the change after the first illumination, although the bands formed by the first illumination obscured the detailed features of the processes. The second illumination experiment confirmed that the band built up around 650 mμ in the dark after illumination is due to P 650 and the leaves are still biologically active for the formation of chlorophyll. Separate experiments showed that both young and normal leaves are active for at least 4 hours in the spectrophotometer. There is a fundamental difference between the effects of the first and the second illuminations, which will be explained later with other data.

Difference spectra generally do not tell us the exact absorption maxima of the bands due to the overlapping of positive and negative bands. However, to see the qualitative changes in the dark processes more clearly, difference spectra were calculated from some of the data for young leaves. Curve A in Fig. 8 was obtained by subtracting curve B from curve C in Fig. 6. It has a positive band at 670 mμ and negative bands at 690 mμ and 640 mμ. We can see the change in the first step, the transformation of protochlorophyll *a* to a substance absorbing at 684 mμ, P 650 $\xrightarrow{\text{light}}$ C 684, in this spectrum. Curve B in Fig. 8 represents the second step, the change between 10 and 60 minutes in the dark (curve F minus curve C in Fig. 6). The maximum positions are 683 and 650 mμ, and the minimum position between these positive bands is 668 mμ. In some samples (see curve D in Fig. 10), this minimum appears as a negative band.

From the experiments in this section on both normal and young leaves, the following facts are clear:

(1) First, the compound that is photochemically produced by the first step has a definite absorption maximum at 684 mμ. To simplify further discussion, this compound is designated as C 684.

(2) In the second step, C 684 is transformed into another compound, C 673, which has an absorption band at 673 mμ in normal leaves and at 671–672 mμ in young leaves. Since we can see an isobestic point over a certain period, there must be a single major compound produced in this step. The small variation of the absorption maximum of C 673 in different leaves is probably due to other factors. The subsequent processes probably effect the apparent absorption maximum of C 673. If the third step and the formation of protochlorophyll proceeds appreciably before the second step ends, the apparent absorption maximum of C 673 will be longer than the true value.

(3) The third step is more complicated, since the formation of protochlorophyll and the shifting of the band of C 673 are proceeding at the same time. The products of this shifting process are designated as C 677. In this particular case, the name may not yet specify a single compound, until we learn more about this compound. The ambiguity comes from the lack of an isobestic point. If the lack of a sharp isobestic point shows that the process is not a single transformation, the formation of C 677 could possibly be a polymerization process, which Krasnovskii *et al.* (6) proposed. But, the simultaneous formation of protochlorophyll during this process might obscure an isobestic point. If this is the case, C 677 is a single compound. The variation of the maximum of C 677 can be explained by the different relative ratio of C 677 to the P 650, simultaneously being formed and to some C 673, being left. A result, which will be explained in the section on second

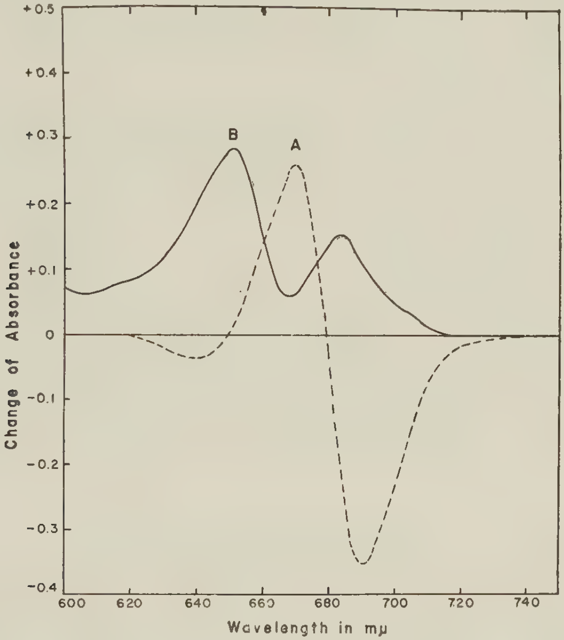


FIG. 8. Difference spectra calculated from the curves in Fig. 6. Curve A, curve C minus curve B in Fig. 6: Curve B, curve F minus curve C in Fig. 6.

illumination, seems to support the latter point of view. Another question remains owing to the absence of an isobestic point; that is whether C 677 is formed from C 673. It could be produced from something other than C 673, but, this is less likely, because, in some cases, we observed a decrease of the band of C 673 during the third step.

(4) If the absorption band of chlorophyll *a* in green leaves or green algae is not shifted by the overlapping of the other bands, C 677 should be chlorophyll *a* in the final natural state, independent of whether it is in a single state, or whether it is composed of different states of chlorophyll *a*. We can not yet draw a definite conclusion as to the specific nature of C 677.

(5) The contradictory results by Smith *et al.* (*3*) and by Krasnovskii *et al.* (*6*) on the shift of the absorption band in the dark after illumination are now easily understandable, if they were observing two different processes; Smith *et al.* measured the transformation of C 684 to C 673 by the second step and Krasnovskii *et al.* saw the formation of C 677 by the third step.

(6) To avoid the confusion in the discussion of the remaining data, the steps shown in this paragraph are summarized in the following schemes, in which the absorption maxima in normal leaves are written first, and when they are different, the maxima in young leaves follow in parentheses. For the formation of C 677, two broken lines indicate two possible ways of its formation.

Preparatory Step

$$\text{Precursor of protochlorophyll } a \xrightarrow{\text{dark}} \text{Protochlorophyll } a,\ \text{P 650}$$

(During the time that the third step is observable, there is also the formation of protochlorophyll *a*. This process was designated as preparatory step to distinguish it from the third step.)

First Step

$$\text{Protochlorophyll } a,\ \text{P 650} \xrightarrow{\text{light}} \text{C 684}$$

Second Step

$$\text{C 684} \xrightarrow{\text{dark}} \text{C 673 (671–672)}$$

Third Step

$$\begin{tikzcd} \text{C 673} \arrow[dr, dashed, "\text{dark}"] & \\ & \text{Final form of chlorophyll } a.\quad \text{C 677 (674–676)} \\ \text{Unknown compounds} \arrow[ur, dashed, "\text{dark}"'] & \end{tikzcd}$$

3. The Absorption Maxima of Extracted Pigments

A simple extraction experiment was carried out in order to obtain the absorption maxima of C 684 and C 673. Immediately after the illumination of young leaves (1000 foot-candles, 1 minute), one half of the sample was ground with ethyl ether and filtered to obtain an extract of C 684. The other half of the sample was kept in the dark for 10 minutes, and was extracted in the same way to obtain an extract of C 673. The absorption spectra of these extracts were measured in the range between 600 and 750 mμ. Both extracts had an absorption maximum at 663 mμ, which is identical with the absorption maximum as chlorophyll *a*, C 677 in this solvent. Owing to the overlapping of the high absorption by carotenoids, these solutions could not be profitably investigated in the Soret region.

This result means that C 684 and C 673 could be either chlorophyll *a* or a compound very similar to chlorophyll *a* or a compound, that can be easily converted into chlorophyll *a* in the process of extraction. Since C 684 and C 673 have different spectra in the plant but in ether are both identical with chlorophyll *a*, it is probable that the change from one to the other is a change *in vivo* in the nature of the combination of chlorophyll *a* with other substances, presumably protein. More extensive studies such as with chromatographic purification will be necessary, before we can say with complete confidence that both are chlorophyll *a* but in different states of combination.

4. Changes of the Spectra in the Soret Region by Illumination with White Light

The difference spectra over the whole visible region were observed to determine the spectrum changes in the Soret region in order to correlate the changes in this region with those, which have been observed in the red region. Two normal etiolated bean leaves were used in both the sample and the reference beams. Since we could not obtain identical samples for both beams, the difference spectrum obtained with non-illuminated samples in both beams did not give us a straight line. This deviation from a straight line depends upon slight differences between the pigment contents of the two samples. This spectrum was used as the zero line for the calculation of the difference spectrum changes. After measuring the difference spectrum of the two non-illuminated samples, the leaves in the sample beam were illuminated by 1000 foot-candles of white light for 1 minute. After letting the samples stand in the dark for certain periods, successive measurements of the spectrum were carried out. The results shown in Figs. 9 and 10 were obtained by subtracting one spectrum from another. Therefore, the calculated spectra in these figures do not include the difference between leaves for the sample and the reference beams. They show only the change of the spectrum of the leaves in the sample beam.

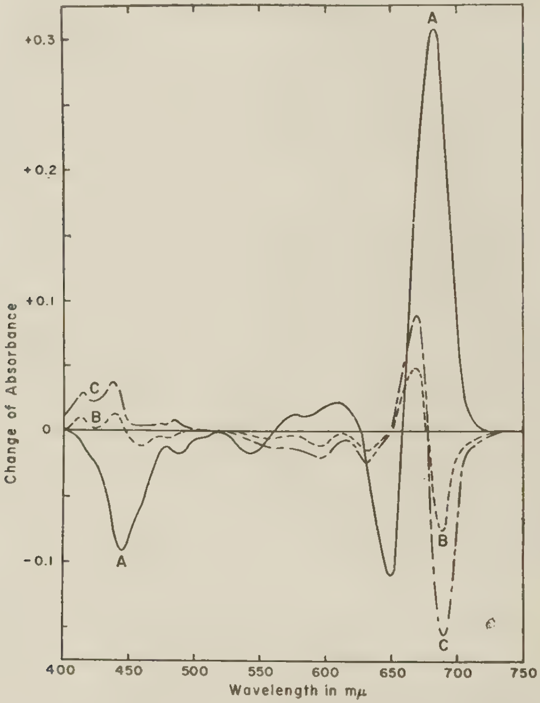


Fig. 9. Difference spectra of two superimposed normal etiolated bean leaves in the visible region (illumination: 1 minute, 1000 foot-candles, white light). Curve A, thes pectrum (1 minute light) minus the spectrum (no illumination): Curve B, the spectrum (1 minute light +15 minutes dark) minus the spectrum (1 minute light): Curve C, the spectrum (1 minute light +60 minutes dark) minus the spectrum (1 minute light).

Curve A in Fig. 9 indicates the change of the spectra caused by the illumination. The positions of the bands in Figs. 9 and 10 are listed in Table I, where maxima or minima refer to peaks or valleys without regard to the zero line. Thus, the positions of negative peaks are written in the row for minima. The small letters, n and p after the numbers indicate whether it is found on the negative or the positive side of the zero line. On curve A, the highest positive peak is at 684 mμ. Its position is not shifted by overlapping of the negative band of P 650. There are two low positive bands at 575 and 610 mμ. The minimum position between these bands is 590 mμ, which must be due to the reduction of the second band of protochlorophyll *a* at 590 mμ. Therefore, the existence of two bands at 610 and 575 mμ appears to be caused by a single positive band near 590 mμ, split into two by the negative band of protochlorophyll *a* at the same position. This interpretation is supported also by the following calculation. The absorption maxima of C 684 and C 673 were calculated, assuming that there is a constant

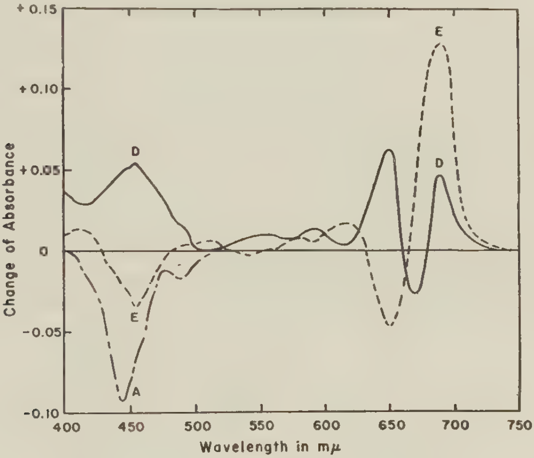


FIG. 10. Difference spectra of two superimposed normal etiolated bean leaves in the visible region; the effect of a second illumination (1 minute, 1000 foot-candles, white light). Curve A, a reproduction of curve A from Fig. 9: Curve D, the spectrum (1 minut light +220 minutes dark) minus the spectrum (1 minute light +60 minutes dark): Curve E, the effect of a second illumination; the spectrum (1 minute light +220 minutes dark +1 minute light) minus the spectrum (1 minute light +220 minutes dark).

shift in wavenumber units between the absorption maximum of C 684 or C 673 *in vivo* and the corresponding maximum of chlorophyll *a* (C 677) in ethyl ether. The constant for the shift was calculated on the difference between 663 and 684 mμ for C 684 and 663 to 672 mμ for C 673. The result is listed in Table II as λ cal. From the calculation shown in this table, we see evidence for the existence of a third band of C 684 at 590 mμ, which supports the above interpretation.

TABLE I

Maximum and Minimum Positions in the Difference Spectra in Figs. 9 and 10

Fingure and curve		Wavelength in mμ
9 A	Max.	575 (p), 610 (p), 684 (p)
	Min.	445 (n), 460 (n), 490 (n), 510 (n), 545 (n), 590 (p), 650 (n)
9 B and 9 C	Max.	414 (p), 440 (p), 572 (n), 616 (n), 670 (p)
	Min.	428 (p), 458 (n), 480 (n), 560 (n), 597 (n), 632 (n), 690 (n)
10 D	Max.	455 (p), 550 (p). 594 (p), 650 (p), 690 (p)
	Min	570 (p), 616 (p), 670 (n)
10 E	Max.	580 (p), 616 (p), 690 (p)
	Min.	455 (n), 540 (n), 650 (n)

TABLE II

Comparison of Calculated and Observed Absorption Maxima of C 684 and C 673

Chlorophyll *a* in ethyl ether	C 684		C 673	
ob.	cal.	ob.	cal.	ob.
663	684	684	672	672
614	632	632	622	616
574	590	597	581	572
431	440	458	435	440

We know another band of protochlorophyll *a*, P 650, at 555 mμ from Fig. 1. In this difference spectrum, there is a negative band at 545 mμ, which is too large to be the change of the 555 mμ band of protochlorophyll *a*. It is therefore probably due to the decrease of both protochlorophyll and the reduced form of some cytochrome. The positive band at 575 mμ might shift this 545 mμ band from its real position.

The greatest decrease in the Soret region is at 445 mμ and there are three low negative bands at 460, 490 and 510 mμ. The position of 445 mμ agrees with the position of the Soret peak in the action spectrum in the transformation of protochlorophyll *a* of Koski *et al.* (*10*). Therefore, the negative band at 445 mμ must be due to the decrease of the Soret band of protochlorophyll *a*, P 650. No positive band appeared in this region. Possibly, the great decrease of the Soret band of protochlorophyll *a* might overcome the increase of the Soret band of C 684, since it is known that the Soret band of protochlorophyll *a* is very high *in vitro* (10). The position, 440 mμ calculated in Table II as the Soret band of C 684 also supports the idea that they are overlapping rather closely. The heights of three low negative bands varies from sample to sample. The significance of the band at 460 mμ will be discussed later.

Curves B and C in Fig. 9 show the changes of the spectra between 0 and 15 minutes and between 0 and 60 minutes in the dark after illumination, respectively. Curve B represents the changes of the second step, while curve C includes changes due to the third step and some protochlorophyll formation. As is shown in Table I, the overlapping effect shifts the band positions of C 684 and C 673 to 690 and 670 mμ. The changes between 650 and 550 mμ show the complication of the overlapping of the decrease of the second and third bands of C 684 and the increase of the bands of C 673. With the aid of Table II, we can find the positive and negative bands corresponding to the second and the third bands of C 684 and C 673.

In the Soret region, there are two positive and two negative bands. The negative band at 458 mμ and the positive band at 440 mμ is likely due to the decrease of C 684 and the increase of C 673, respectively. It is not clear whether the other bands at 480 and 414 mμ are related to C 684 and C 673 or not. Since illumination may shift the equilibrium states of other compounds than the chlorophylls in etiolated leaves, the bands at 414 and 480 mμ may have arisen from other substances.

The change after 60 minutes is shown in curve D in Fig. 10, which is the difference spectrum between 60 and 220 minutes in the dark after illumination. In the red region, we can see the formation of protochlorophyll *a* at 650 mμ, the reduction of C 673 at 670 mμ and the formation of C 677 at 690 mμ. The slight difference of these maxima from the corresponding values obtained before can be accounted for by the overlapping effect in difference spectrum. The greatest increase in the Soret region appears at 455 mμ. The formation of protochlorphyll *a* is shown only as a shoulder near 445 mμ. The band at 455 mμ is probably due to C 677.

5. *Effect of a Second Illumination*

The above sample kept in the dark for 220 minutes was reilluminated in the same way as before (1000 foot-candles, 1 minute). The difference between the spectra before and after the illumination is shown in curve E in Fig. 10. For comparison, the difference spectrum (curve A) in the Soret region due to the first illumination is reproduced in the same figure. There is a striking difference between the spectra of the first and second illuminations especially in the Soret region. Instead of the main decrease of the protochlorophyll *a* band at 445 mμ found after the first illumination, a band at 455 mμ decreased on the second illumination. The decrease of the 445 mμ protochlorophyll a band appeared only as a shoulder. A further interesting feature of the change is that curve E looks like the mirror image of curve D with a mirror at a certain horizontal level in the Soret region. This indicates that the substances produced by the third step are reduced by the second illumination. This suggests the possibility that there may be corresponding differences also in the red region. In order to see these changes in the red, the difference spectra of the first and second illuminations were calculated from the spectra shown in Figs. 6 and 7, which were observed on the same young leaves using the expanded wavelength scale in the red region. The results are shown in Fig. 11, where the scale for these curves is indicated on the left side of the figure. Curves A and B are the difference spectra before and after the first and second illumination, respectively. Curve A has a positive band at 684 mμ, while curve B has it at 687 mμ. This difference can not be accounted for by the overlapping effect, since we have relatively the same decrease of the band at 650 mμ. In order to see the difference between these curves more distinctly, curve C was calculated by subtracting curve A from curve B. The scale for curve C is indicated on the right side of the figure. Curve C has a positive band at 643 mμ and a sharp negative band at 679 mμ. The problem is whether this curve represents the difference only between the amounts of protochlorophyll transformed on the first and second illuminations, or whether additional processes are superposed on the transformation of protochlorophyll *a*. Actually, the positive band on curve C is too round to be a band of protochlorophyll *a*. The position is also different from 650 mμ.

To make sure that curve C does not indicate the excess amount of protochlorophyll *a* transformed, the following test was made. Before subtracting curve A from curve B, the absorbance of curve A was multiplied by an arbitrary constant, in order to check the possibility that the subtracted curve could be a straight horizontal line. Trials with several constants showed that the smoothest and most reasonable curve was

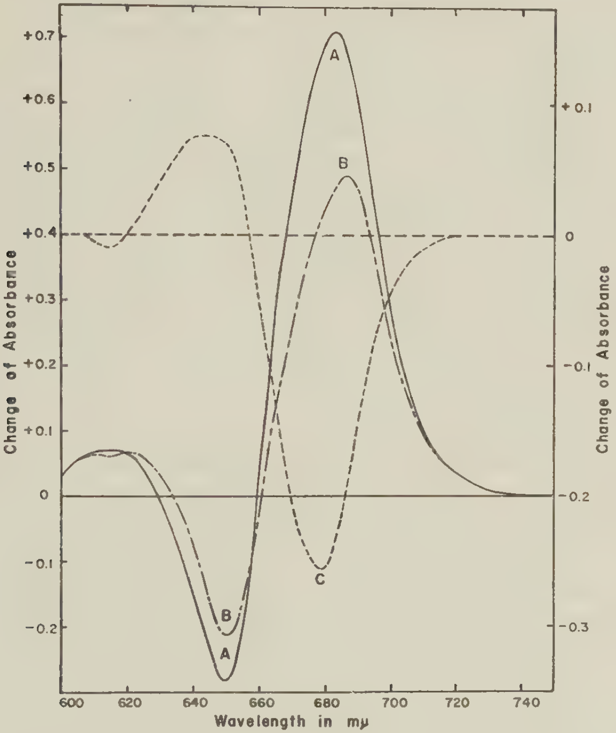


FIG. 11. Differences between the effects of the first and the second illuminations in the spectra in Figs. 6 and 7. Curve A, difference spectrum caused by the first illumination (curve B minus curve A in Fig. 6): Curve B, difference spectrum caused by the second illumination after 100 minutes in the dark following first illumination (curve H minus curve G in Fig. 7): Curve C, curve B minus curve A in this figure.

obtained without any factor or with the constant, unity. Once a constant is multiplied into curve A, a sharp negative or positive peak appears at 650 mμ, being superposed on the round positive peak of curve C. As further evidence, curve A was subtracted from the difference spectrum of the second illumination of a different sample with no factor multiplied into curve A. In this case, a sharp peak of protochlorophyll *a* was observed on the subtracted curve.

These calculations confirmed the conclusion that curve C does not indicate a difference of the amount of protochlorophyll *a* transformed, but rather that curve C represents another process caused by the second illumination in addition to protochlorophyll transformation. Furthermore, the amounts of protochlorophyll *a* transformed by both illuminations are the same. Since the sample consisted of the same leaves for both illuminations and it was kept in the dark until the formation of protochlorophyll *a* stopped, it is quite reasonable to have the same amount of protochlorophyll *a* before these illuminations. Probably, there is a limited reservoir for protochlorophyll *a*, which is filled in the dark after an adequate time lapse.

We know now that a compound, which has an absorption band near 679 mμ, is decreased by the second illumination as well as the decrease of protochlorophyll *a*. We also know that this compound has a Soret band at 455 mμ and is accumulated in the third step. Therefore, it is almost certain that it is C 677, if we admit the slight difference of the absorption maximum, being due to an overlapping effect.

It is worth while to point out two important properties of C 677. This C 677 is responsible for the absorption maximum at 676–678 mμ in green leaves, and furthermore it is capable of being decreased photochemically. This implies that C 677 may itself undergo a chemical change in the photochemical step of photosynthesis. The participation of C 677 to photosynthesis is stressed by the recent observations of Coleman, Holt and Rabinowitch (*17*). They observed a change of absorption in the red region of the spectrum caused by illumination of a *Cholorella* suspension. Their difference spectrum showed a negative band at 680 mμ. They think that it corresponds to the reversible reduction of chlorophyll *a in vitro* observed by Krasnovskii (*18*) and Evstigneev and Gavrilove (*19*), and also corresponds to ionization of enolized chlorophyll investigated by Weller (*20*). The position of the maximum is very close to the maximum of C 677, although the change in height in our case is greater by a factor of 100 than the change they observed. If these two phonomena are the same, then more illumination experiments with slightly greened etiolated samples may well be the key to solve the photochemical step of photosynthesis, because a much greater change is observable in slightly greened leaves than in mature plants.

Secondly, the behavior of C 677 may support the proposal of Virgin (*21*). According to his results and separate experiments by Tolbert and Gailey (*17*), the production rate of chlorophyll *a* is greatly enhanced after about two hours' illumination of etiolated leaves. According to Smith (*22, 23*) it is about the same time when oxygen evolution by illuminated etiolated leaves is greatly enhanced. The existence of the different phase is also supported by the observation of radioactive CO_2 fixation by illuminated etiolated leaves by Tolbert and Gailey (*24*). Virgin (*21*) proposed that the mechanism of chlorophyll formation is different during the rapid production phase from the mechanism at the beginning. As is clear from our experiment, two hours are required for the formation of an appreciable amount of C 677. It may be that C 677 may play a role in the formation of chlorophyll *a* in the rapid production phase that Virgin has found.

If chlorophyll *a* is formed by a different mechanism in the rapid production phase, the question arises as to whether the formation of chlorophyll *a* is being carried out in this phase through protochlorophyll *a*. As was mentioned before, protochlorophyll *a* is reformed even after the second illumination. But, the conditions of preillumination followed by a second illumination after a dark period are not natural and the reformation of protochlorophyll *a* could be a minor process as compared with the other processes, if the formation of chlorophyll *a* is not formed through protochlorophyll *a* in the rapid phase.

In order to solve this problem, an experiment was carried out with two etiolated bean leaves, which were kept under room light of about 10 foot-candles for two hours. The leaves illuminated in this way were placed in the spectrophotometer, and the change of the spectrum was followed in the dark. Curves A, B and C in Fig. 12 show the change of the spectrum between 0–15, 0–30 and 0–60 minutes in the dark after illumination. The formation of protochlorophyll *a* starts just after the illumination and the rate is very rapid, especially at the beginning. Another similar experiment with room light of 100 foot-candles also showed the same rapid production of protochlorophyll *a* immediately after the illumination, although in this case the increase in absorption over the whole red region prevented the quantitative evaluation of the rate. This over-all increase may have been due to an increase of scattering. In the spectra in Fig. 12, the second step is observed as the shift of a band from 683 to 680 mμ. There is a hump around 710 mμ. In this stage of the experiment, the role of this band is not clear. It is very likely, however, that this small band corresponds to the additional negative band beyond 700 mμ in the difference spectrum of Coleman *et al.* (*17*).

Nevertheless, the above experiment demonstrates that the refor-

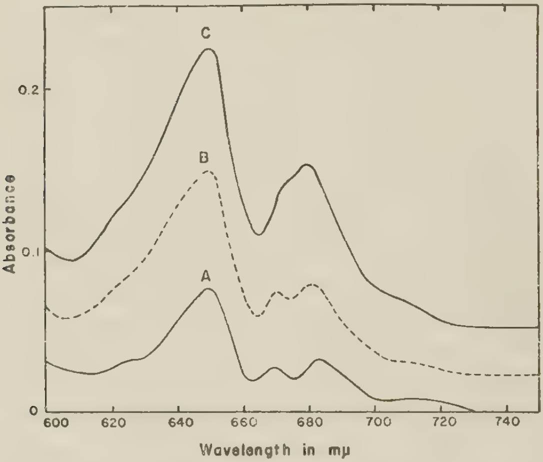


Fig. 12. The formation of protochlorophyll *a* in leaves kept for two hours under room light of 10 foot-candles, then darkned. Curve A, the spectrum (2 hours room light +15 minutes dark) minus the spectrum (2 hours room light +0 minute dark): Curve B, the spectrum (2 hours room light +30 minutes dark) minus the spectrum (2 hours room light +0 minute dark): Curve C, the spectrum (2 hours room light +60 minutes dark) minus the spectrum (2 hours room light +0 minute dark).

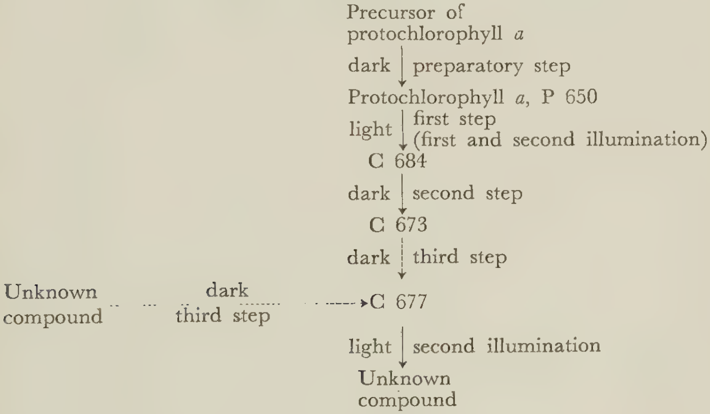
mation of protochlorophyll starts at a rapid rate at the beginning of darkness after two hours' illumination. This fact confirmed that chlorophyll *a* could be formed through protochlorophyll *a* in the rapid phase, even if the mechanism is different.

6. *Changes of the Spectra caused by Illumination with Monochromatic Light*

The changes of the spectra of etiolated leaves by white light are naturally occurring processes. As was shown so far, they are rather complicated. With the hope of being able to separate or identify some of the processes more clearly, the monochromatic light of 650 mμ was used for the illumination of the samples. Red light of 650 mμ is the light which is mainly absorbed by protochlorophyll *a*. Similar experiments with this red light did not give us any apparently different results from that by white light. All phenomena observed after white light occurred in a similar way in the changes of the spectrum in the dark after the illumination by red light. Therefore, it is certain that the main dark processes effected by white light are caused by the absorption of light by protochlorophyll *a*. The fact that only the protochlorophyll *a* band appeared in the action spectrum by Koski *et al.* (*9*) agrees with this result.

CONCLUSIONS

It was found in this study that the transformation of protochlorophyll *a* into chlorophyll *a* is not a simple process. The process can be summarized in the following way.



Once etiolated leaves are illuminated, many processes are initiated. The process proceeds in a series of reactions. In the second illumination, the photochemical transformation of C 677 initiates another series of reactions, although we only know the first step. In this study, only the changes of colored compounds were followed, and some of the steps found could be the superposition of several steps. More thorough studies by the spectroscopic method combined with chemical investigation will unravel the complex process, which perhaps includes the initial steps of photosynthesis.

At any rate, this study has clearified the controversy on the shifting of the band formed from protochlorophyll *a* after illumination, and also showed that the preparation obtained by Smith *et al.* (*3*) is protochlorophyll *a* holochrome in his sense. The correlation with continued studies *in vitro* on the steps defined here *in vivo* will add much to the final solution on the mechanisms of chlorophyll formation.

The plants used for this study were grown by Drs. J.H.C. Smith and D.W. Kupke. The author wishes to express his hearty thanks for their kindness in supplying the experimental materials for this investigation and also for their guidance and valuable discussions. The optical instruments for the illumination of the samples were bult by Dr. C. S. French, whom the author wishes to thank for his constant advice and encouragement during this research.

SUMMARY

1. It has been found possible to record clearly and on a large scale the absorption spectrum of protochlorophyll and its photochemical transformation products in living etiolated leaves. By this method, the kinetics of chlorophyll formation have been investigated *in vivo*.

2. Protochlorophyll *a*, which can be transformed by light to chlorophyll *a*, has its red absorption peak at 650 mμ. There is also present a form of protochlorophyll *a* with a peak at 636 mμ, which is inactive. These substances, differing from each other *in vivo* have been designated as P 650 and P 636.

3. It was found that the immediate product of illumination of an etiolated leaf has its red absorption peak at 684 mμ. This purely photochemical effect has been designated as step one and the product formed has been called C 684.

4. The second step, a dark reaction, transforms the photochemical product, C 684, to one that has its peak at about 673 mμ, called C 673. This reaction is nearly completed in about 10–20 minutes.

5. The third step, also a dark reaction but slower than the second step, changes C 673 to a substance with a peak at about 677 mμ, which is characteristic of chlorophyll *a* in living mature leaves under natural conditions. In this period, the formation of protochlorophyll *a* can be observed.

6. Leaves containing C 684 or C 673 when extracted give spectra

in ether identical to ordinary chlorophyll *a*, C 677.

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TRANSAMINASES IN A HALOPHILIC BACTERIUM NO. 101

II. PHENYLALANINE-GLUTAMIC ACID TRANSAMINASE

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In the previous report (*1*) the author described that clear cell-free extracts of a halophilic *Pseudomonas* strain No. 101 showed strong phenylalanine- and aspartic acid—α-ketoglutaric acid transaminase activities. On the basis of different behaviors during the heat-treatment of the enzyme extracts and during the growth of the cells it was suggested that these two activities would be ascribed to two different enzymes. In the present paper the author reports on some of the further results obtained with phenylalanine-glutamic acid transaminase, regarding zone-electrophoretic fractionations, balance of the reaction, and the equilibrium constant.

METHODS

Preparation of Cell-free Extracts—Halophilic *Pseudomonas* strain No. 101 was grown on a shaker at 30° in 500 ml. flasks containing 100 ml. of medium composed of Ajinomoto's "Mieki" (amino acids containing solution prepared from the soybean meal HCl- hydrolysats, 20 g.-*N*/1), 10 per cent; NaCl, 10 per cent, at pH 7.2. After 48 hours the cells were harvested by centrifugation and washed twice with 10 per cent saline. Extracts were prepared by treating a thick paste of cells with distilled water. The clear supernatant solution obtained by centrifugation of the water-lysed material at $20,000 \times g$. for 30 minutes was used as the source of the enzyme. Such extracts contained 0.2 to 1.2 mg. of nitrogen per ml.

Test for Transaminase Activity—For testing transaminase activity, reaction mixtures usually contained 0.2 ml. of enzyme, 50 μg of pyridoxal phosphate, 50 μM of L-amino acid, and 30 μM of α-keto acid in a final volume of 1 ml. of 0.1 *M* phosphate buffer at pH 7.0. The mixtures were incubated in stoppered tubes at 37° for desired periods of time. Variations in this procedure are given in case of need.

Paper Chromatographic Estimation of Glutamic Acid, Phenylalanine, α-Ketoglutaric Acid, and Phenylpyruvic Acid—The method used by us includes modifications of other published methods (*2, 3, 4*). The reaction was stopped by the addition of 0.1 ml. of 95 per cent formic acid, and 0.01 ml. of the reaction mixture was spotted on Toyo No. 51 filter paper sheets of approximately 40 × 40 cm. After drying, the paper was transferred to a chamber containing 200 ml. of water-saturated *n*-butanol-formic acid (95:5) and the solvent allowed to ascend at room temperature until it had moved about 18 cm. For determining α-ketoglutaric acid and phenylpyruvic acid, the papers were then sprayed heavily with the semicarbazide reagent (0.1 per cent semicarbazide in 0.15 per cent sodium acetate solution). After drying, the paper were examined under ultraviolet light. The semicarbazones appeared as dark shadows against the light background due to the faint fluorescence of the paper. For the quantitative determination of the keto acids present, rectangular sections containing the semicarbazones under examination were cut out and placed in the test-tubes after being dissected into pieces. The size of the sections was the same for all samples and filter paper blanks. For the colorimetric determination, 0.5 ml. of the 2,4-dinitrophenylhydrazine solution (0.025 per cent solution of the reagent in $M/2$ HCl) was added to each tube, followed by 5 ml. of 10 per cent KOH after 20 minutes at 37°. The tubes were then shaken gently for 10 minutes to permit extraction of the hydrazones which had been formed within the paper itself. Readings of the intensity of color were made against a filter paper-reagent blank at 435 $m\mu$.

For determining glutamic acid and phenylalanine, chromatograms were developed by spraying lightly 0.1 per cent ninhydrin in butanol saturated with water and they were dried in an oven at 100° for 5 minutes. The colored spots of amino acids were cut into small squares and placed in the test tubes. After removing NH_3 by the addition of 0.5 ml. of 0.1 N NaOH and evacuation, one ml. of ninhydrin reagent (2 per cent ninhydrin and 0.3 per cent hydrindantin in methyl cellosolve-4 N sodium acetate buffer (3:1), pH 5.5) were added to each tube, and the tubes were then heated in a covered boiling water bath for 15 minutes. After cooling, the contents of the tubes were filled up to 10 ml. with *n*-propanol-water (1:1) and the color intensity was read on a spectrophotometer at 570 $m\mu$ with a blank prepared from filter paper containing no amino acid.

Enzymatic Method for Determining L-*Glutamic Acid*—L-Glutamic acid was determined by the slightly modified manometric method of Umbreit and Gunsalus (*5*). The reaction was stopped by placing the tube in boiling water for 5 minutes, the pH was adjusted to 4.8, and 1 ml. thereof was transferred to the main cup of a Warburg vessel containing 1 ml. of 0.2 M acetate buffer, pH 4.8. Three tenth ml. of a 30 mg./ml. suspension of dried cells of *E. coli* " Crookes " strain containing the specific decarboxylase was added to the side arm. After 10 minutes equilibration at 30°, the contents of the side arm were tipped in and the evolution of CO_2 was followed until the decarboxylation was completed.

Zone Electrophoresis—Zone electrophoresis was carried out with potato starch as the supporting medium in an apparatus similar to that of Kunkel and Slater (*6*). The commercial potato starch was washed thrice with three times its volume of 0.05 N NaOH. The alkali was removed by washing with water, which was followed by several washings with the buffer solutions to be employed. Starch for the solidification of input material was obtained by drying starch following the water wash. The runs were made at 0° in buffer of $M/20$ concentration. The crude enzyme solution in 1.5 ml. of buffer corresponding to 1 mg. N was introduced into a 1 cm. slit cut crosswise in a 40×2×1 cm. block of the starch. The strip of the block was then cut crosswise into sections 2–3 cm. wide, each of which was extracted with 1–1.5 ml. of distilled water. Finally, analyses for protein and measurement of activity were made on suitable aliquots of the extracts. Protein was determined by the modified method of Folin.

RESULTS

Results obtained with the cell free extracts, when these were subjected to electrophoresis in Tris buffer at pH 8.4 for 7.5 hours at 320 volts, are presented in Fig. 1 in which are plotted the relative value of enzyme activities and protein concentration of eluate as a function of the distance from the starting trench. The pattern of distribution of enzyme activity indicates the presence of at least two distinct enzyme components, one of which was completely separated from the others. Further electrophoretic studies showed that the resolution of the components was not improved when tested in phosphate buffers at pH 6.2. The specific activities and activity ratios of the eluates in the peak regions are given in Table I. These values are to be compared with the ratio

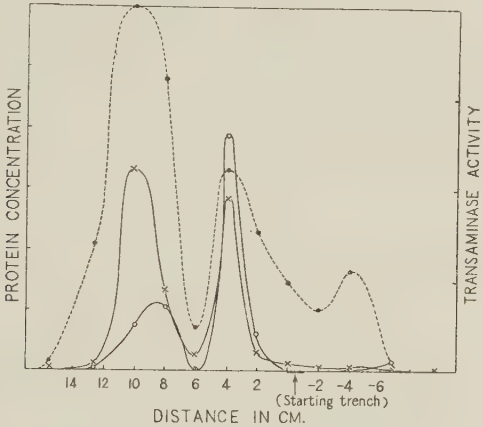

Fig. 1. Distribution of protein (······), phenylalanine- (–×–×–) and aspartic acid- transaminase activities (–O–O–) in starch zone electrophoresis of crude transaminase preparation at pH 8.4 ($M/20$ Tris buffer) for 7.5 hours at 320 volts.

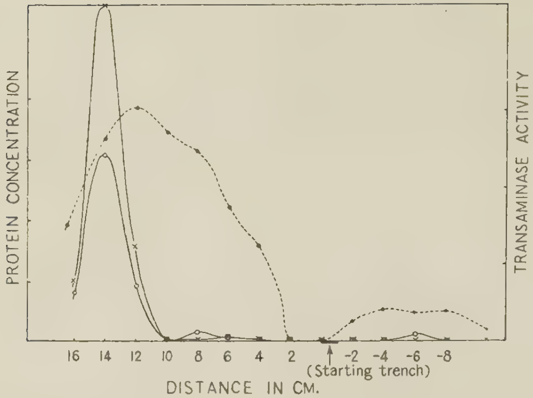

FIG. 2. Distribution of protein (……), phenylalanine- (-O-O-) and aspartic acid- transaminase activities (-O-O-) in starch zone electrophoresis of crude transaminase preparation at pH 6.2 (*M*/20 phosphate buffer) for 8 hours at 320 volts.

TABLE I

*Specific Transaminase Activity and Activity Ratio of Some Fractions in Starch Zone Electrophoretic Separation**

Conditiones in electrophoresis	Position of fraction in cm. from origin	Transaminase activities (Q_{TN})		Activity ratio (A)/(P)
		Aspartic acid (A)	Phenylalanine (P)	
pH 8.4 Tris buffer, 320 v. 7.5 hrs.	4	536	392	1.35
	10	63	270	0.23
pH 6.2 phosphate buffer, 320 v. 8 hrs.	12	300	515	0.58
	14	1,170	2,120	0.55
	16	534	667	0.80

* Reaction mixture contained 0.20 ml. of enzyme, 30 μM of α-ketoglutaric acid, 50 μM of L-amino acid as indicated, and 50 μg. of crude pyridoxal phosphate in a final volume of 1.0 ml. of 0.1 *M* phosphate buffer at pH 7.0. Incubated 3–4 hours at 37°. N content of enzyme fractiones was calculated from Folin-optical density and experimental factor. (N=O.D. ×0.236 mg. *N*/ml.)

between aspartic acid- and phenylalanine—α-ketoglutaric acid transaminase activity of the input material: 0.56.

Transamination Balance—Table II shows the results of studies on transamination balances. The enzyme used was crude cell-free extracts similar to the one described above. In the reaction between L-phenylalanine and α-ketoglutaric acid, 1 μM of each of the substrates disappeared while 1 μM of each of the products, glutamic acid and phenylpyruvic acid, was formed.

TABLE II

*Transamination Balance**

Substrate	Reaction time	Phenylalanine μM/ml. found	change	α-ketoglutaric acid μM/ml. found	change	Glutamic acid μM/ml. found	change	Phenylpyruvic acid μM/ml. found	change
Phenylalanine + α-ketoglutaric acid	0 *hr.*	24.6	0.0	29.2	0.0	0.0	0.0	0.0	1.0
	2	19.6	−5.0	24.0	−5.2	4.2	4.2	4.1	4.1
	4	17.2	−7.4	21.0	−8.2	7.5	7.5	6.3	6.3
	8	15.5	−9.1	19.8	−9.4	10.1	10.1	8.1	8.1
	14	14.1	−10.5	18.5	−10.7	10.5	10.5	10.2	10.2
Glutamic acid + phenylpyruvic acid	0	0.0	0.0	0.0	0.0	23.8	0.0	19.4	0.0
	2	2.4	2.4	3.2	3.2	21.5	−2.3	15.8	−3.6
	4	3.7	3.7	4.4	4.4	19.9	−3.9	14.7	−4.7
	8	6.4	6.4	7.7	7.7	16.8	−7.0	11.3	−8.1
	14	9.8	9.8	10.2	10.2	14.5	−9.3	9.1	−10.3

* Reaction mixtures consisted of 0.2 ml. of enzyme (0.19 mg. *N*/ml.), 50 μg. of crude pyridoxal phosphate, and substrates as given in the table in a final volume of 1.0 ml. of 0.1 *M* phosphate buffer at pH 7.0. Incubated at 37°.

A similar balance was obtained when the reverse reaction was studied. These data provide evidence that these are true transamination reactions.

If the reactions were initiated from both side of equilibrium point, the value, (α-ketoglutarate) (L-phenylalanine)/(phenylpyruvate (L-glutamate), should increase or decrease to the same equilibrium constant. As can be seen from column 6 of Table III, the equilibrium constant for the glutamate-phenylalanine reaction:

Table III

Determination of Equilibrium Constant at 37° *and pH* 7.0*

Reaction time, hr.	α-Ketoglutaric acid (KG), μM/ml.	Phenylpyruvic acid (Ppv), μM/ml.	Glutamic acid (Glu), μM/ml.	Phenylalanine (Phe), μM/ml.	$\frac{(KG)\,(Phe)}{(Ppv)\,(Glu)}$
0	0.0	37.1	29.9	0.0	0.0
20	16.9	22.9	12.9	16.2	0.928
0	20.9	13.9	16.3	14.2	1.31
5	22.3	13.0	16.3	14.2	1.49
0	38.3	0.0	0.0	30.0	∞
10	22.3	15.0	14.9	14.7	1.47

* Conditiones were similar to that of above table II.

$$K = \frac{(\alpha\text{-ketoglutarate})\ (\text{L-phenylalanine})}{(\text{L-glutamate})\ (\text{phenylpyruvate})}$$

was found to be approximately 1.5 at pH 7.0 and at 37°.

DISCUSSION

In the previous paper it was suggested that the strong phenylalanine- and aspartic acid—α-ketoglutaric acid transaminase activities of this bacterium would be ascribed to two different enzymes from the different behaviors during the heat-treatment of the enzyme extracts and during the growth of the cells. This concept was further confirmed by the present studies on the two transaminase activities of fractions obtained by zone electrophoresis.

A recent determination by Krebs (*7*) of the equilibrium constants gave a value of 1.52 at pH 7.4 and 25° for the glutamate-alanine reaction. The present determination of the equilibrium constant gave a value of 1.5 at pH 7.0 and 37° for the glutamate-phenylalanine reaction. Therefore, this result indicates that the introduction of phenyl group to β-position of alanine does not affect the free energy change in the transamination reaction.

It appeared possible that the phenylalanine—glutamic acid transaminase of this bacterium can be utilized for the preparation of L-phenylalanine from phenylpyruvic acid. Actually, crystalline L-phenylalanine hydrochloride was obtained by a modified method of Partridge (*8*) from the reaction mixtures with 92 per cent yield. The detailed procedures and results on this point will be published elsewhere.

SUMMARY

Zone electrophoresis of the crude transaminase preparation, which is almost specific to aspartic acid and phenylalanine as amino donors, has indicated the multiple nature of the enzyme system.

From balance studies of the reactants and demonstration of the reverse reaction, it was confirmed that phenylalanine-glutamate reaction catalysed by cell-free extracts of halophilic *Pseudomonas* strain No. 101 was a true transamination reaction.

The equilibrium constant for the glutamate-phenylalanine reaction was found to be approximately 1.5 at pH 7.0 and 37°.

The author is indebted to Prof. S. Akabori for the helpful criticism and encouragement during the course of this work, to Prof. F. Egami of the Nagoya University, for the gift of the bacterial strain, to Mr. H. Mitsui and Miss. T. Wada for the helpful advices concerning zone electrophoretic and paper chromatographic techniques respectively, and to Mr. M. Shiroishi of the Food Research Institute, the Department of Agriculture and Forestry, and Mr. S. Sakurai of the Osaka University for the gift of pyridoxal phosphate and phenylpyruvic acid respectively.

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A NEW MICRODETERMINATION OF THE TOTAL QUANTITY OF CYSTEINE PLUS CYSTINE IN PROTEIN BY HYDRAZINOLYSIS

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A review of the literature indicated a number of methods for the estimation of cystine or cysteine in protein, amperometric (*1*), phosphotungstic acid (*2*) and sulfonic acid methods (*3*) etc. with denaturants of protein or reducing reagents of cystine, but none appeared entirely satisfactory for the determination of the total quantity of cystine plus cysteine because of its complicated procedures and intricate reactions between reagents and protein.

In this paper we describe a new simplified and cystine- and cysteine-specific method of determination by applying hydrazinolysis which was originated by S. Akabori and K. Ohno (*4*, *5*).

Both cystine and cysteine undergo reductive decomposition with hydrazine at 120°, giving rise to hydrogen sulfide specifically and quantitatively. It was confirmed that from methionine and taurine no hydrogen sulfide was produced by hydrazinolysis in the same conditions. The possible reactions are given as follows:

$$\text{Cystine}+\text{hydrazine}\rightarrow 2H_2S+NH_3+N_2+\text{unknown substance}$$

Hydrogen sulfide produced is determined by colorimetric method utilizing Caro's reaction (*6*, *7*) in which methylene blue is formed.

EXPERIMENTAL

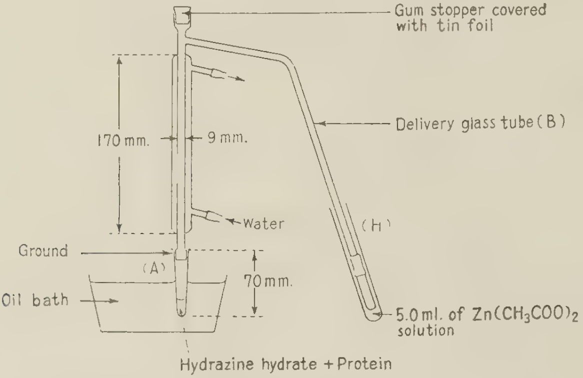
Reagents

Purified Hydrazine Hydrate—Distilled and dehydrated hydrazine with calcium oxide (*8*) is added with equi-molar distilled water and kept in sealed tubes in 0.5 ml. portions.

Zinc Acetate Solution—60 g. of zinc acetate, 17 g. of sodium acetate and 0.05 g. of sodium chloride is dissolved in distilled water and diluted to 1 liter.

Ferric Ammonium Sulfate Solution—31 g. of ferric ammonium sulfate is dissolved in distilled water and 6.3 ml. of concentrated sulfuric acid is added. The volume of solution is made to 250 ml. with water.

Dimethyl-p-phenylenediamine Hydrochloride Solution—0.5 g. in 1 liter water solution containing 200 ml. of concentrated sulfuric acid.



Gum stopper covered with tin foil
170 mm.
9 mm.
Delivery glass tube (B)
Water
(H)
Ground
(A)
70 mm.
Oil bath
5.0 ml. of $Zn(CH_3COO)_2$ solution
Hydrazine hydrate + Protein

FIG. 1. Apparatus for hydrazinolysis.

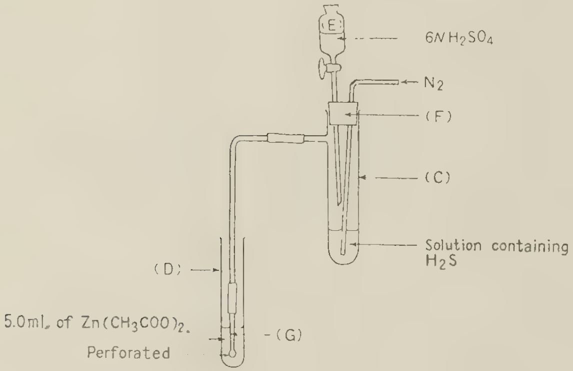


FIG. 2. Apparatus for the formation of zinc sulfide from hydrogen sulfide produced by hydrazinolysis.

6N Sulfuric Acid.

25.0 μ moles (6.00 mg.) of L-*Cystine as Standard Material.* (Corresponding to 50.0 μ moles of hydrogen sulfide).

Preparation of calibration curve of hydrogen sulfide.

The calibration curve for hydrogen sulfide was prepared according to the method described by L. H. Almy (*7*) applying iodometry and using hydrogen sulfide solution as standard, but now it can be done by our method employing L-cystine as standard.

A protein sample containing about 10 to 50 μ moles of cystine plus cysteine is placed in the apparatus (A) (Fig. 1). 0.5 ml. of hydrazine hydrate is added, and then equipping condensor and delivery glass tube (B), (A) is heated at 115 to 120°. After five to seven hours (the time of heating can be shortened by decreasing the quantity of protein), (A) is cooled with cold water and the condensor is washed down with 2 or 3 ml. of distilled cold water, and then content of (A) is removed to volumetric flask, being diluted so that 4 or 5 ml. of the diluted solution contains 1 to 3 μ moles hydrogen sulfide. 4.0 or 5.0 ml. of the solution is removed into test tube (C) of the apparatus (Fig. 2). After sealing (C) with gum stopper (F) tightly, 10 ml. of 6 N sulfuric acid is runned into (C) from funnel (E), the flow being stopped in time to prevent the last few drops from leaving the bulb. Nitrogen gas is passed through for 30 minutes from gas holder or bomb at rate of flow 1.2 to 1.5 liter per thirty minutes. It is necessary to heat (C) in a water bath at 50° to 55° to drive out hydrogen sulfide completely, which is absorbed with 5.0 ml. of zinc acetate solution placed in the test tube (D). After aeration the glass tube (G) connected to delivery tube with rubber tubing is taken away and put into zinc acetate solution.

5.0 ml. of dimethyl-*p*-phenylenediamine solution and 1.0 ml. of ferric ammonium sulfate solution is rapidly pipetted into the zinc acetate solution in this order, which is gently agitated after each addition closing with rubber stopper tightly. The colored solution is sometimes stirred by shaking the test tube.

After one hour, the colored solution is diluted five- or tenfold with distilled water and estimated by a photoelectric colorimeter with 10 mm. cuvette and filter of 630 mμ. which shows maximal light absorption.

RESULTS

The blank test using hydrazine hydrate solution did not color the zinc acetate solution and gave no differences between hydrazine hydrate and distilled water as sample for blank when the colored solution was diluted to tenfold. Zinc acetate solution placed in the test tube (H) was not colored by Caro's reaction. This fact shows that hydrogen sulfide is retained in hydrazine hydrate solution even at 120° for seven hours.

L-*Cysteine and* L-*Cystine*—Six examples are given in Table I. The calculations are based on the reactions described above. It is noticeable that the quantities of hydrogen sulfide produced from L-cystine or L-cysteine are agreed with the values calibrated from the standard curve which is prepared from the solution of hydrogen sulfide by iodometry, that is, 1 mole of L-cystine produces 2 moles of hydrogen sulfide and

1 mole of L-cysteine produces 1 mole of hydrogen sulfide.

L-*Methionine*—This substance contains sulfur, but it does not produce hydrogen sulfide with hydrazine hydrate. Though L-methionine and amino acids were added, the recovery of hydrogen sulfide produced from L-cystine was quite satisfactory (Table II). Those results make our methods possible to determine the hydrogen sulfide derived from

TABLE I

Determination of L-Cystine and L-Cysteine

Calculated			Experimental	
L-Cystine*	L-Cysteine*	H_2S producible	H_2S estimated	Error
μ moles		*μ moles*	*μ moles*	%
24.7		49.4	50.0	+1
24.7		49.4	48.9	−1
12.5		25.0	24.6	−2
25.5		51.0	52.5	+3
	24.3*μ moles*	24.3	23.9	−2
	22.6	22.6	22.1	−2

* Heated for 5 to 7 hours.

TABLE II

Interference of L-Methionine and Other Amino Acids in Recovery

		H_2S produced	Recovery
(1)	L-Methionine 50 *μ moles*	none	
(2)	L-Cystine 25.2 *μ moles*+L-Methionine 50 *μ moles*	49.4 *μ moles*	98 %
(3)	L-Cystine 26.4 *μ moles*+L-Methionine 49 *μ moles* +*Hydroxy-L-Proline+L*-Tyrosine +*L-Lysine +L*-Proline +*DL-Tryptophane+L*-Histidine +*L-Arginine +L*-Phenylalanine	53.0	100

* 2 or 3 mg. of each amino acid was added.
(1), (2), (3)—heated for 7 hours at 115° to 120°.

cystine and cysteine in protein.

Determination of Cystine in Lysozyme—The lysozyme recrystallized four times and lyophilized was thoroughly dried by Abderhalden drying apparatus in the presence of phosphorus pentoxide at 78°. The hydrogen sulfide produced from 29.8 mg. of the dried lysozyme was equivalent to 5 moles of cystine contained in one mole of lyzozyme assuming that molecular weight of lysozyme is 14,900 (Table III).

The value of cystine is quite agreed with those which have been reported using phosphotungstic acid method (*9*) or calculations (*10, 11*). G. L. Mills (*12*) has reported somewhat larger value of 5.7 moles by DNP method.

TABLE III

Determination of Cystine in Lysozyme

Lysozyme	Castine estimated	Cystine per 1 mole of lysozyme
*2.00 μ *moles*	9.85 μ *umoles*	4.93 *moles*
*2.10	10.7	5.08

* Heated for 5 to 7 hours.

TABLE IV

Determination of Half-cystine in Amylase (α-type)
Prepared from " Takadiastase Sankyo "

Amylase	Half-cystine determined	Half-cystine per 100 g. of amylase	Half-cystine per 1 mole of amylase
mg.	μ *moles*	*g.*	*moles*
*37.5	1.46	2.33	10.5
*37.5	1.42	2.27	10.2

* Heated for 6 to 8 hours.

Determination of the Total Quantity of Cystine plus Cysteine in Crystalline Amylase (α-Type) Prepared from " Taka-diastase Sankyo "—The protein recrystallized three times and was dried as in the case of lysozyme. The values obtained do not consist with that which has been reported by Akabori *et al.* (*13*) by means of phosphotungstic acid method (Table IV).

Crystalline Amylase (α-Type) Prepared from Bacillus subtilis—Cystine or cysteine is not contained in the enzyme protein by our method. K. Sugae (*14*) has reported that this enzyme protein has methionine as one of the constituent amino acids but not cystine or cysteine by cysteic acid method, etc. Accordingly, the fact that production of hydrogen sulfide is not found in decomposition of the enzyme protein proves hydrogen sulfide is not formed from methionine in protein.

Determination of the Total Quantity of Cystine plus Cysteine in Ovalbumin Recrystallized—It was reported by R. Tristram (*15*, *16*) that 2 moles of half-cystine and 5 moles of cysteine are contained in ovalbumin. 7.46±0.09 moles of half-cystine was found in one mole of ovalbumin by our method (Table V) assuming that molecular weight of the protein is 46,000.

Comparison of Reductive and Decomposing Action of Anhydrous Hydrazine with That of Hydrazine Hydrate—The remarkable decrease of formation of hydrogen sulfide from lysozyme with anhydrous hydrazine was observed, while the difference between the two decomposing agents was not considerably found in the decomposition of L-cystine (Tables VI & VII.

TABLE V

Determination of Half-cystine in Ovalbumin

Ovalbumin	Half-cystine determined	Half-cystine per 100 g. of ovalbumin	Half-cystine per 1 mole of ovalbumin
mg.	*μ moles*	*g.*	*mole*
*51.8	8.50	1.97	7.55
*25.7	4.13	1.93	7.37

* Heated for 5 to 7 hours.

Commercial Hydrazine Hydrate—Commercial hydrazine hydrate of 80 per cent concentration was used in the decomposition of lysozyme, and it was quite satisfactory to produce hydrogen sulfide quantitatively in the conditions described above.

Comparison of Decomposing Action of Sodium Hydroxide with Hydrazine Hydrate—In our experiments 1.0 ml. of 6 *N* sodium hydroxide was used, but it could not completely decompose lysozyme to produce hydrogen sulfide quantitatively though we decreased the quantity of lysozyme to one-tenth (3 to 4 mg.). A reductive agent would be required besides alkali solution.

Ammonium Sulfate and Taurine—Ammonium sulfate is used in the preparation of protein and taurine is a sulfonic acid existing in organism but hydrogen sulfide was not produced from those substances.

TABLE VI

Decomposition of Methionine and Cystine with Anhydrous Hydrazine

L-Methionine	(*μ moles*)	40					
L-Cystine	(*μ moles*)		24.2	23.8	24.8	24.9	25.0
Anhydrous hydrazine	(0.5 *ml.*)	+	+	+	+	+	
Hydrazine hydrate*	(0.5 *ml.*)						+
Time of heating	(*hours*)	5	2	5	2	5	2.5
Per cent estimated		0	47	98	52	97	64*

* Compare with Table I.

TABLE VII

Decomposition of Lysozyme with Anhydrous Hydrazine

Lysozyme	(*mg.*)	31.2	29.8	30.2
Cystine calculated	(*μ moles*)	10.5	10.0	10.1
Anhydrous hydrazine	(0.5 *ml.*)	+	+	
Hydrazine hydrate	(0.5 *ml.*)			+
Time of heating	(*hours*)	5	5	5
Per cent of cystine estimated, calculated according to the data described in Table III.		53	51	99

SUMMARY

A new method for the microdetermination of the total quantity of cystine plus cysteine in protein by hydrazinolysis was described. It was confirmed that hydrogen sulfide was produced specifically and quantitatively from cystine or cysteine. The total quantity of cystine plus cysteine in some proteins was determined.

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ON THE N-TERMINAL RESIDUES AND SEQUENCE OF CLUPEINE FROM CLUPEA PALLASII: THE OCCURRENCE OF PROLINE IN ADDITION TO ALANINE AS THE N-TERMINUS*

Sirs :

It was reported by some of the present authors that all our specimens of clupeine prepared from *Clupea pallasii* always have alanine as the N-terminal residue (*1*, *2*), while clupeine from *Clupea harengus* was reported by European authors to have only proline. Further careful experiments on our less soluble clupeine (fractionated by making use of the solubility difference of its picrate) have now confirmed that not only alanine, but proline too occupy the terminal to various extents and that both the 2nd and the 3rd residues are occupied by arginine. The conclusion has been derived from the following results.

(1) DNP-Clupeine was digested with crystalline trypsin for 10 min., 6 and 40 hours. The methanol extracts of the dried digest were chromatographed on talc column. Free peptides were easily washed away with *N*-HCl while DNP-peptides were remained strongly adsorbed. Then the first eluate of the DNP-peptides with EtOH-*N*-HCl (1 :4, v/v) was found to contain DNP-prolyl peptides which gave the same absorption in ultraviolet as DNP-proline in *N*-HCl (*3*) and yielded only proline (*ca.* 1 mole/mole of DNP-group), dinitrophenol and arginine after their complete hydrolysis (6*N*-HCl, 110°, 24 hours). The proline and the dinitrophenol must have occurred from the DNP-proline at the terminal under the condition as indicated by a control experiment on DNP-proline. The second eluate with EtOH-*N*-HCl (4 :1, v/v) contained DNP-alanyl peptides which yielded DNP-alanine and arginine on hydrolysis. In some cases, preceding talc chromatography, continuous paper electrophoresis was employed to separate DNP-peptides containing one molecule of arginine and two (estimated by the color values of Sakaguchi reaction per DNP-group). DNP-Pro-Arg, DNP-Ala-

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* A part of this paper was read by one of the authors (M. Y.) before the Symposium on Protein Structure, Tokyo, Nov. 4, 1955.

Arg, DNP-Pro-Arg-Arg and DNP-Ala-Arg-Arg were thus isolated. In each case, the content of proline at the N-terminal was estimated from the absorption at 375 mμ of the DNP-prolyl peptides. It was *ca.* 10 per cent for a specimen of clupeine obtained in 1947 (at Mombetsu facing the Sea of Okhotsk, Hokkaido, Japan), while it was *ca.* 50 per cent for that obtained in 1951 (at Yoichi facing the Japan Sea, Hokkaido, Japan).

(2) The ratio (designated as R) of an optical density at 390 mμ to that at 360 mμ has a characteristic value for absorption curves of DNP-amino acids and -peptides (*4*). The values of R of DNP-alanine and DNP-alanyl peptide (λ_{max} always at *ca.* 353 mμ) were *ca.* 0.55 in *N*-HCl, while those of DNP-proline and DNP-prolyl peptide (λ_{max} always at 375 mμ) *ca.* 1.02. From the measurement of absorption curves in *N*-HCl of the mixtures of DNP-alanine and DNP-proline in various proportions, the relation of the calculated values of R to the contents of DNP-proline was found to be linear (Fig. 1). According to the figure, a specimen of DNP-clupeine prepared from the material obtained in 1947 (R=0.67) was calculated to contain *ca.* 25 per cent of DNP-proline as the N-terminal, whereas that in 1951 (R=0.83) *ca.* 60 per cent of DNP-proline. DNP-Salmine, in contrast, has R value of *ca.* 1.02 and was confirmed to be a DNP-prolyl peptide. DNP-Pro-Arg and DNP-

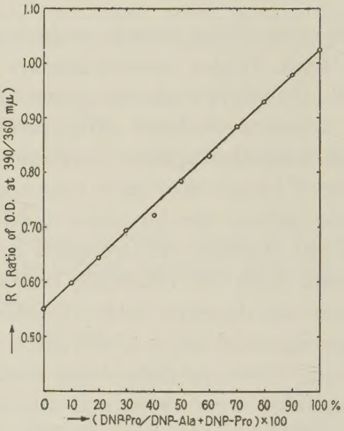

FIG. 1. The relation between the composition of the mixture (DNP-Ala+DNP-Pro) and the ratio of optical densities at 390/360 mμ of absorption curves of the mixture.

Pro-Arg-Arg were isolated from the latter by method 1.

(3) An ethereal extract from the acid hydrolysate (6 *N*-HCl, 100°, 8 hours) of DNP-clupeine was passed through a bufferized celite column consisting of two layers (the upper was bufferized with 1 *M*-phosphate buffer of pH 5.4 and the lower with 0.4 *M*-one of pH 6.5) (*5*). DNP-Alanine and dinitrophenol separated in this way were estimated by making use of their absorption at 360 mμ in 4 per cent $NaHCO_3$ solution. When corrected for control experiments, 0.4 and 0.45 mole of DNP-alanine and dinitrophenol, respectively, were obtained from 1 mole of DNP-clupeine (1951), while 0.7 mole of DNP-alanine (*i.e.* less than 0.3 mole of DNP-proline) from another specimen (1947). As the control, hydrolysis of DNP-alanine, DNP-alanine+clupeine, DNP-proline, and DNP-salmine was performed with recovery (for experiment of 8 hours-hydrolysis) of 98 (as DNP-alanine), 96 (DNP-alanine), 88 (dinitrophenol), and 69 per cent (dinitrophenol), respectively. DNP-Protamines used here were purified previously through a column of Amberlite IRC-50 (XE-64) and then with IRA-400 to be completely free from dinitrophenol.

Although many reasons, such as the heterogeneity of clupeine molecules, mixed species of the material, or some changes in the material after the capture, may be considered to explain the above result on the N-terminal residues of the present specimens of clupeine, it is not clear which is the most probable case. Details will be fully reported later. When this manuscript has been finished, an interesting paper by Scanes and Tozer was published (*6*) which showed alanine as the N-terminus in addition to proline in a specimen of clupeine, though the species of its origin was not described.

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